BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

FINAL REPORT

P-4483/G-2

(NO. 41-10246)

Work on this project was conducted under the auspices of Canada Centre for Mineral and Energy Technology, Energy, Mines and Resources, Canada.

DSS FILE NO.: 26SQ.23440-3-9137
DSS CONTRACT SERIAL NUMBER: OSQ 83-00197
CANMET PROJECT NUMBER: 350204
CANMET SCIENTIFIC AUTHORITY: Mr. H.W. PARSONS

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April 17, 1985

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BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

EXECUTIVE SUMMARY

1. INTRODUCTION

The Ontario Research Foundation was awarded a contract in response to an unsolicited proposal to evaluate "The Biological Control of Thiobacillus ferrooxidans by Predatory Organisms."

In Phase I of the program, the objective was to undertake a critical assessment of the published literature to assess the feasibility of using predatory organisms to control the numbers of viable T. ferrooxidans in tailings and hence inhibit the production of acid mine drainage by the action of these autotrophic micro-organisms.

In Phase II of the program, the objective was to investigate the use of one type of predator selected on the basis of the information obtained in Phase I.

2. GENERAL OVERVIEW

Acid mine drainage is considered to originate predominantly from the metabolic activity of the autrophic, iron-oxidizing bacterium, T. ferrooxidans. Control technologies to prevent the growth of this organism within tailings include physical storage methods to create an anoxic environment and may involve vegetation cover, capping, or deep-water disposal. Such methods are sensitive to environmental factors including rainfall (leaching), evaporation, erosion and seismic activity which may lead to restoration of ecological conditions favourable to the growth of acid-generating bacteria. As long-term disposal methods, these storage procedures have many weaknesses.

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Biological control methods have many potentially attractive features. The agents are isolated from the natural environmental and hence do not introduce a foreign substance. A desirable attribute would be their ability to be self-maintaining within a tailings pile.

3. PHASE I - LITERATURE REVIEW

Control mechanisms assessed included:

a) Active predators for T. ferrooxidans including rotifers, ciliated or flagellated protozoa.

b) Parasitic or predatory bacterial species such as Caulobacter spp.or Bdellovibrio spp.

c) Production of metabolites (by a micro-organism) which are toxic to T. ferrooxidans.

d) Isolation of a bacteriophage specific to T. ferrooxidans.

e) Prevention of the lowering of pH within tailings such that T. ferrooxidans will not actively grow (i.e., it requires a pH of less than 4.0).

None of the methods assessed was considered sufficiently well developed for immediate application. Based on the available data, predatory organisms were recommended for further investigation at the laboratory level. Rotifers and protozoa can cause significant reductions in bacterial numbers over relatively short time intervals and can withstand relatively severe environmental stresses such as dehydration, shortage of nutrients, low or high temperatures, acidity, etc.

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4. PHASE II - LABORATORY PROGRAM

Methods were developed to isolate organisms able to utilize T. ferrooxidans as prey. Sources of these organisms included samples of tailings and acid drainage from the Nordic Tailings (Elliott Lake) and samples of potential predatory species were received from another laboratory. Local freshwater samples were also investigated.

Two cultures of protozoa were selected for further study, one able to survive at pH greater than 4.0 and one active at acidic pH values (pH 2.5 - 3.5). Both of these mixed, unidentified protozoan cultures utilized T. ferrooxidans cells as prey in suspension cultures. However, survival of the predators in viable forms appeared to be dependent an adequate number of T. ferrooxidans cells being present in a suspension. These cultures were selected based on factors such as physical size, (i.e., smaller organisms preferred) and hardiness, (i.e., resistance to factors such as pH and oxygen depletion likely to prevail at lower zones of tailings piles where microbial acid generation is most prevalent).

The more acidophilic protozoan culture was selected for evaluation of its effect on the acid-generating ability of pyrrhotite tailings in controlled laboratory tests using lysimeter boxes.

The results of these preliminary evaluations were not conclusive due to the limited period and scale of the test. A single application of a predator culture did not inhibit acid generation. Multiple applications of predator culture over an interval of two weeks appeared more promising but needs further investigation. Some inhibition of acid generation was observed in the test lysimeter boxes. A similar effect was also observed in the control boxes (no predator added). Active protozoa were found to survive within the tailings. However, the predominant species, large ciliated protozoa, were not observed in the predator cultures applied to the tailings. Similar types of ciliates were observed in the control columns indicating that they may have been dormant within the tailings as received. Alternatively, they may have been introduced in the make up waters which were not sterile.

5. CONCLUSIONS AND RECOMMENDATIONS

1. The preliminary program has identified several important factors for which current information is inadequate for the development of a technology to control acid mine drainage by biological agents.

2. Methods were developed to isolate organisms able to utilize T. ferrooxidans as prey.

3. Several ciliated protozoa and zooflagellate cultures obtained from Dalhousie University did not survive through transit.

4. Protozoan cultures were isolated which were able to use T. ferrooxidans cells as prey.

5. The physical size of the organisms and the particle size distribution within tailings are identified as important parameters liable to influence the ability of predators to penetrate and survive through tailings porewater.

6. Dormant protozoan cultures may exist in acid tailings pile.

7. A hierarchy of predatory cultures may develop within the tailings pile.

8. Due to the dynamic equilibria that can exist between predators and prey, supplementary nutrition through bacterial cultures other than T. ferrooxidans or other nutrients may be necessary.

9. Although the results of initial studies are inconclusive potential predators and mechanisms are identified.

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10. Larger preparations of predatory organisms should be prepared and their effect evaluated in large scale lysimeter boxes. Noticeable changes in seepage quality can then be more easily identified, due to the large volumes of predator and T. ferrooxidans cells in seepage solutions.

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BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

PHASE I

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ORF Report P-4483/G-2

BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

1. INTRODUCTION

This report is submitted in accordance with the terms of DSS Contract Serial Number OSQ 83-00197

2. OBJECTIVE

This program is directed at the application of biotechnology to the control and prevention of acid drainage from mine tailings. The objective of Phase 1 has been to undertake an in-depth evaluation of the published literature on biological interactions which, in the natural environment, will inhibit the growth of Thiobacillus ferrooxidans and hence the generation of acid mine drainage. The technical feasibility of these potential biological agents will be assessed and those organisms and/or techniques of interest will be recommended for study in Phase 2.

3. LITERATURE SEARCH

A computer-assisted literature has been done and appropriate references have been assembled. The literature search has been conducted in two stages. In the first stage, specific biological agents or methods to control acid mine drainage have been sought. The keywords used and the data bases searched are listed in Appendix I. This provided a very limited number of relevant papers and was indicative of the level of development of the technology. In the second stage of the literature search, we concentrated on examining the characteristics of known predatory or parasitic organisms relative to their potential application in the ecological environment of a tailings pile. The feasibility of using these organisms to control the population of T. ferrooxidans is assessed in later sections. In this group was also included the use of techniques to control acid generation by inhibiting the metabolic processes of one or more bacterial species involved in the overall reaction mechanism by which acid is generated from pyritic minerals.

4. BACKGROUND

4.1 Acid Mine Drainage

Acid mine drainage is considered to originate predominantly from the metabolic activity of the autotrophic, iron-oxidizing bacterium, T. ferrooxidans. The ability of this organism to oxidize ferrous iron and reduced sulphur compounds with the production of acidic ferric sulphate solution has been well documented. This acidic solution initiates the oxidative dissolution of sulphide minerals, especially iron pyrite, in ore residues and tailings. T. ferrooxidans can then maintain the dissolution reaction, and the acidity of the environment, by its continued catalysis of the ferrous/ferric oxidation reaction. The result is a serious problem due to the escape of highly acidic effluents into streams and lakes. Often, toxic metals will be leached from the ores by the acid.

During the past two decades, control technologies have received a great deal of attention. Many control methods are based on chemical treatments to neutralize and render insoluble any potentially toxic elements. The tailings must then be stored in a manner to discourage or prevent the growth of T. ferrooxidans. Physical storage methods to create an anoxic environment may involve vegetation cover, capping or deep-water disposal. These methods, however, are sensitive to environmental factors such as rainfall (leaching), evaporation, erosion, seismic activity, etc. Their action may result in the restoration of ecological conditions favourable to the growth of acid-generating bacteria in the tailings. As long-term disposal methods, these storage procedures have many weaknesses.

In light of these limitations for chemical or physical techniques, biological control methods have many attractive features. The agents are themselves isolated from the natural environment and, therefore, do not introduce a foreign substance. By establishing a controlled biological population within a tailings heap (as the tailings are generated), it is hoped to create a self-generating mechanism to prevent the excessive development of acid-generating bacterial communities.

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The control mechanisms will involve either the destruction of T. ferrooxidans cells as they reproduce or the creation of an environment which will not support the growth of this organism even when a suitable substrate is present. These agents or approaches include:

a) Active bacterial predators for T. ferrooxidans such as rotifers, ciliated or flagellated protozoa.
b) Parasitic or predatory bacterial species such as Caulobacter or Bdellovibrio.
c) Production of metabolites (by a microorganism) which are toxic to T. ferrooxidans.
d) Isolation of a bacteriophage specific to T. ferrooxidans.
e) Prevention of the gradual lowering of pH within tailings such that T.ferrooxidans will not be active i.e. it requires a pH of less than 4.0.

In our review, we have attempted to include all possible routes to the biological control of the acid-generation pathway. Some consideration has also been given to the ecological and geological factors which will influence the survival of both the acid-generating and the control organisms.

4.2 The Ecology of a Tailings Pile

In current management practices, the tailings, as generated by the mill, may be either slightly below neutral or alkaline. The exact characteristics will depend on the wastewater treatment technologies in use at a mine. If lime is applied to remove metals and neutralize acids, the alkaline sludge will be discharged usually with the tailings. The constituents of the residual ore particles will have a certain ability to neutralize acid. This will depend on the mineralogical composition of the original ore. The ability to sustain the growth of T. ferrooxidans will depend on the presence of reduced iron and reduced sulphur compounds in the tailings e.g. iron pyrite (FeS_2). This too will be dependent on the chemical composition of the original ore.

The gradual generation of an acidic environment in a tailings heap will be assisted by the leaching action of rainwater. The level of acidity in the rainfall at different regions of the continent will vary and hence the rate at which the neutralization occurs, may be significantly greater in certain localities.

Biological organisms require moisture for growth and survival. The small particle size of tailings will facilitate the retention of water at the particle surfaces as well as in the extensive interstitial spaces. Microorganisms also require a food supply. In the case of the autotroph T. ferrooxidans, there is usually no lack of nutrients i.e. reduced iron and sulphur minerals, in tailing piles. This bacterium is aerobic and during autotrophic growth obtains carbon for the generation of cellular components from atmospheric carbon dioxide. The necessary oxygen and carbon dioxide will be supplied in part by the amounts of these gases dissolved in the rainwater. During periods of low rainfall, upper zones of the tailings will dry out which will permit air to penetrate to subsurface regions.

It has been proposed that the sequence of reactions leading to the creation of an environment favourable for the growth of acid-generating bacteria, such as T. ferrooxidans, may involve other iron-oxidizing microorganisms. Thus, a succession of bacterial communities, each catalyzing a specific stage of the overall reaction sequence, will lead eventually to the establishment of an acid-generating culture. Singer and Stumm (1970) showed that the rate of generation of acid by the dissolution of iron pyrite in coal is dependent on the rate of ferrous iron oxidation. Stumm and Lee (1961) had previously found that the rate of non-biological oxidation of ferrous iron is directly dependent on the solution pH. At pH 3.5-4.5, however, the rate is independent of solution acidity (Singer and Stumm, 1970). The ferric iron produced by these reactions will oxidize iron pyrite.

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T. ferrooxidans requires a pH of less than 3.5 for good growth and at that value the catalytic effect of the organism on the $Fe^{2+} \rightarrow Fe^{3+}$ reaction is significant (Schnaitman et al., 1969). Walsh and Mitchell (1972a, 1972b) identified an iron-oxidizing bacterium of the genus Metallogenium which, they found, would efficiently oxidize ferrous iron in the pH range 3.5-4.5. They proposed that this biologically mediated oxidation of iron would accelerate the slow, abiotic oxidation process in this pH range. They hypothesized that this would create the acidic conditions favourable for the growth of T. ferrooxidans. In their tests, the presence of Metallogenium in culture medium (pH 4) inoculated with T. ferrooxidans led to an active acid-producing culture (pH 2.6) within 45 days, whereas culture medium inoculated with only T. ferrooxidans remained at pH 4 with no detectable growth of the organism.

Walsh and Mitchell (1972b) concluded that the inhibition of Metallogenium activity should interrupt the succession of iron-oxidizing bacteria and prevent the rapid development of acid-generating communities. Further discussion of their investigations will be presented in a later section.

The development of acid-generating cultures in tailings will also be influenced by the presence of organic residues, toxic metals and other constituents from the ore-processing plant. Although, these constituents may inhibit the growth of T. ferrooxidans, the eventual release/mobilization of these toxic components will usually lead to the restoration of conditions able to support the growth of the organism. A process of natural selection may also lead to the presence of metal-tolerant strains.

4.3. A Strategy for Biological Control

A successful biological control agent or method must be able to maintain an ecological environment within the tailings which will inhibit, over a long period of time, the development of a large population of acid-generating bacteria. Two basic approaches are either

a) the inoculation of a predator for T. ferrooxidans

or

b) the creation of an environment which will not support the growth of T. ferrooxidans.

In the ideal situation, the former approach will be a form of "bacteriostatic" agent. If the rate of predation is too great, the predator will deplete its environment of bacterial cells, leading to its own demise. For long-term control of the situation, the predator should be able to exist in a steady-state with the T. ferrooxidans. Thus, the rate of predation should not exceed the rate of bacterial reproduction but should be able to maintain the microbial population at a non-critical level so that little or no acid production is observed.

In the latter situation, the control agent must, over a long interval, maintain the tailings environment in a condition which is hostile to T. ferrooxidans. If, for example, an approach is based on maintaining the pH value at 5.0 or higher (by the inhibition of Metallogenium), the treatment must also be able to neutralize any acid produced by the abiotic oxidation of ferrous ions to the ferric form which will subsequently precipitate as ferric hydroxide i.e.

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3(ppt) + 3H^+$$

Although this reaction is much slower than the biologically catalyzed production of acid, it may over a long period of time cause the pH within the tailings to drop to a critical level. The development of T. ferrooxidans communities might occur in localized zones of higher acidity.

The effectiveness of either of these control technologies will be influenced significantly by several factors, including the minerology of the tailings; the microecology and interactions between bacteria and particles; the population dynamics of both the endogenous bacteria within the tailings and the inoculated predatorial organisms; the diffusional characteristics of toxic or neutralizing chemicals or metabolites; the moisture balance; the specificity of an agent towards a target organism etc. All of these aspects have been borne in mind during our appraisal of the literature.

5 LITERATURE REVIEW

Our assessment of the literature is divided into two major sections. In the first section, we will report on those techniques which claim to be control technologies. In the second section, we will review some of the fundamental characteristics of known bacterial predators or parasites.

5.1 Biological Control of Acid-generating Organisms Methods and Agents Reported in the Literature

5.1.1 Caulobacter spp.

Shearer et al. (1968) examined samples of the waters from coal mines showing no apparent acid drainage problem. They tested the effect of these waters, which they considered contained natural inhibitory compounds, on the generation of acid by T. ferrooxidans inoculated into a coal pile. Using an upward-flow, column reactor, they found that the inoculation of some non-acidic mine waters caused a significant reduction in the amount of acid produced. Shearer and Everson (1969) attempted to identify the inhibitor(s) present in these waters. Tests for bacteriophage were negative. Caulobacter were identified to be present and several strains of Caulobacter were assessed for their ability to inhibit the growth of T. ferrooxidans (as indicated by the oxidation of iron in specific culture media). These authors also investigated the effect of different types of reactor systems on the inhibition reaction. The best results appeared to occur with an upward-flowing vertical system.

In later studies, Shearer et al.(1970) isolated Caulobacter spp. from the non-acidic mine waters. A strain was adapted to grow in a synthetic acid mine drainage liquor (pH 3.6) and cultured to yield high cell numbers. These Caulobacter cells were diluted and sprayed on to the surface of a settling pond which had iron/sulphur oxidizing bacteria present. The authors stated that after two months the acidity of the effluent from the pond was lower. Although iron and sulphur-oxidizing bacteria were still present, their numbers

appeared to be reduced. At no time were accurate viable cell counts undertaken on the iron-oxidizing population and this raises some doubts about the validity of the above observation.

The assumption that the inhibitory species was biological in origin was based on the results of plate studies using solid nutrient medium (Shearer et al., 1968). They noted an apparent improvement in inhibitory activity towards T. ferrooxidans following a series of enrichment cultures. The methods and experimental techniques were poorly documented.

Baker and Wilshire (1971) queried whether the apparent inhibition of the acid generation was in fact due to the activity of a Caulobacter sp. They obtained a sample of the culture from Shearer et al. and carried out their own series of tests. They hypothesized that the acclimation of a Caulobacter sp. to grow actively at pH 3.6 was inconsistent with known data on this organism. They noted that Shearer et al. did not clearly demonstrate that the T. ferrooxidans cultures in the coal columns had been allowed to reach dynamic equilibrium prior to the inoculation of Caulobacter cells. Shearer et al. inoculated the coal columns with Caulobacter cells in the original, peptone-containing culture medium i.e. the cells were not washed. Baker and Wilshire (1971) stated that the sudden addition of medium may have resulted in washout of the acid-generating organisms on the coal substrate. There would also be a loading of organic plus inorganic components in the Caulobacter culture medium. Baker and Wilshire concluded from their tests that although the Caulobacter sp. remained constant in numbers, there was no evidence of a reduction in the numbers of iron-oxidizing bacteria nor an increase in the pH. Thus the Caulobacter sp. did not appear to be parasitic towards the chemoautotrophic bacteria. Their observations could, however, be challenged in that they presented no evidence from defined tests, that the Caulobacter sp. obtained from Shearer et al. was still active against T. ferrooxidans. This omission leaves open the possibility that the inhibitory activity towards T. ferrooxidans was not a stable quality and had been lost during the subculturing and maintenance of the Caulobacter strain.

Shearer et al.(1968), and Shearer and Everson (1969) obtained their best results with flooded upward-flow reactors. They did test a horizontal flooded bed reactor and although inhibition was observed to occur, it appeared to migrate only slowly through different zones along the reactor.

Baker and Wilshire (1971) used a horizontal flooded-bed reactor with recycle. Shearer and Everson (1969) did not use recycling of leachate with any of their reactor systems. It is difficult, therefore, to compare accurately the operating parameters used by the respective research groups. Factors such as the number of Caulobacter cells per mL of inoculum, the composition of the feed water, etc., may have been significantly different in the two sets of tests.

The conclusions of Baker and Wilshire cast some doubt on the ability of Caulobacter spp. to control acid mine drainage. However, the strategy followed by Shearer et al.(1968, 1969) is noteworthy in that they attempted to identify why certain coal mines did not appear to generate acidic effluents. Because of the lack of data, we have assumed that the acid-generation potentials of the coals was the same in both the acid and non acid-generating coal mines.

The biochemistry and microbiology of Caulobacter spp. will be considered briefly in a later section of this review.

5.1.2 Interference in Microbial Successions

Walsh and Mitchell (1972a) isolated an acid-tolerant, iron-oxidizing bacterium from iron-bearing streams. This filamentous organism was identified as a Metallogenium sp. These authors hypothesized that this bacterium, whose iron-oxidizing activity was greatest at pH 3.5-4.5, was responsible for creating an acidic environment favourable for the development of T. ferrooxidans. . Data were published confirming the apparent ecological role for this Metallogenium sp.(Walsh and Mitchell,1972b). They

reported that the iron-oxidizing activity of the Metallogenium sp. was inhibited by increasing ferrous iron concentrations and subsequently (Walsh and Mitchell, 1975) described a control strategy for inhibition of acid generation in a coal mine based on increasing the ferrous iron concentration in the influent waters. The procedure (using ferrous iron concentrations of 100 ppm) was successful in laboratory tests with columns of coal particles.

Subsequently, Walsh (1980) claimed that using this concept it would be possible to control acid generation by recycling mine water (neutralized to pH 4.5) They contended that the ferrous iron content of the effluent would be sufficient to inhibit the Metallogenium and prevent the T. ferrooxidans succession. It should be noted that this inhibitor will not prevent totally the oxidative weathering of pyrite i.e. chemical mechanisms will still be operative above pH 4.5 but the rates of reaction will be much reduced.

5.1.3 General Overview of Reported Control Technologies

Both of the methods described above are classified as "not proven" since promising data obtained by the original researchers have not been duplicated by other laboratories. In order to expand the knowledge base of these methodologies, we shall review the general characteristics of each type of biological control agent.

6.0 FUNDAMENTAL ASPECTS

In this section we will examine the basic ecology and biology of potential control organisms. This will include predatory organisms such as rotifers and protozoa which were not identified in either of the above control technologies.

Control technologies will use either a predator species to prey on the acid-producing bacteria or the creation of a hostile environment to inhibit a succession of biologically induced chemical reactions. Each of these approaches is complex involving not only the population dynamics of predator-prey relationships or the ecological interactions of bacterial communities present in the environment of tailings particles but also the influence of other variables such as moisture level and temperature.

We shall briefly review each of these topics from the standpoint of control of acid-generating organisms in tailings.

6.1 Predator-Prey Relationships

In this context, we shall consider the development of control technologies for T. ferrooxidans based on the use of rotifers or protozoa as biological agents. In those situations, the predator consumes the prey as a food. For the predator to survive within such an ecology, there must be an adequate supply of the food. Thus, in the case of the ciliated protozoon (biflagellate) isolated by Ehrlich (1963) from acid mine water, the protozoon did not multiply until long after the number of autotrophic bacterial cells had reached a maximum. This may indicate a preference by that predator for dead bacterial cells (Seed, 1971). If this observation is correct, the inability of the predator to consume live bacterial cells would not be consistent with an inhibition of the production of acid.

Starkweather et al.(1979) stated "The conventional view among zooplankton ecologists has been that free living bacteria are seldom the primary constituents in rotifer nutrition while they may serve as supplementary or diversifying food sources in the total diet". Pourriot (1965) is stated by these authors, to have been unsuccessful in maintaining long-term cultures of Brachionus calyciflorus on Bacillus rubens or Aerobacter aerogenes alone. This suggested that the bacteria were of limited nutritional value to the rotifer. However, their own studies (Starkweather et al., 1979) showed that B. calyciflorus could be maintained for over 40 generations on A. aerogenes as food source. These authors noted that the bacterial clearance and ingestion rates were also dependent on the food density.

Curds (1973), using computer models, studied the ecology of sewage bacteria (activated sludge) and carnivorous, ciliated protozoa. These latter protozoa feed on bacteria-consuming ciliates and their existence might prove a threat to any control technology using the application of large amounts of ciliated protozoa to tailings. Thus, all of the bacterial-consuming predators are not only selective as to the bacteria on which they will prey but they are also susceptible to predation themselves by larger ciliates, rotifers etc.

Fenchel (1980b) identified bactivorous ciliates as "opportunists" in that they respond to localized situations involving packets of high concentrations of bacterial cells. Low cell concentrations do not support the growth of these ciliates.

Another factor impinging on the predator-prey balance is the presence of parasites of the predators. It has been shown that bdelloid rotifers are susceptible to fungal parasites (Barron, 1980a; Barron and Tzean, 1981; Barron and Szijarto, 1982).

In any study using a predator of T. ferrooxidans as a control agent, the above factors will come into play. More common factors such as the respective reproduction rates of the predator and prey, the specifity of the predator for a particular prey, the temperature, humidity, and the effect of particulates will also impact on the effectiveness of the method. Many of the data on predator-prey relationships (involving bacteria as prey) have been based on aquatic communities. This is true with respect to the observations on rotifers (Oliver, 1967) and flagellated protozoa (Ehrlich, 1963) in acid mine-waters. Tailings, the environment in which a control technology is intended to be applied, will introduce additional constraints. For example T. ferrooxidans associates intimately with the surfaces of pyritic minerals and it is not known how this will affect predator-prey relationships.

It is difficult to envisage a predator having the ability to eradicate T. ferrooxidans. Numbers may be significantly reduced but the potential for continued acid development by remaining organisms will be ever present. This will also apply where predators are introduced to tailings at pH values above the growth range of T. ferrooxidans. Certainly the process of acid generation may be slowed down but it is difficult to see this extending to prevention of acid formation.

6.2 Microbial Communities

This section covers both the relationships between predatory and parasitic bacterial species, such as Caulobacter spp. and Bdellovibrio spp., with T.ferrooxidans. It will also consider the role of microbial succession in the process leading to acid generation in a tailings pile.

The former group involves many of the same limitations as the larger predator organisms. Generally, one observes predatory species existing in ecological balance with each other. Total eradication of prey species is not normal. Instead one identifies significant and wide fluctuations in the numbers of bacterial prey and their predator. This cyclical pattern,typical of a relationship between predator and prey,can be expected in a tailings environment. Mineral particles will also influence the balance between predator and prey bacteria.

Microbial successions leading to the development of acid generation within a tailings pile are of particular interest in that they may offer a control mechanism operational at higher pH values of 5.0-6.5. Once acid-generation is occurring, biological agents active only in this range will lose much of their value. Harrison (1978) studied an artificial pyritic coal spoil over a period of 1-1/2 years. The coal pile was inoculated with a slurry of acidic soil and drainage water from an old coal strip mine spoil. Unfortunately, the experiment was conducted at a temperature of 27^{o}C and on a relatively small scale. The development of strong T. ferrooxidans growth and a drop in pH to 2.6 occurred in higher regions within 8 weeks. Along the littoral zones (5 cm above the water surrounding the mound), the pH remained at 5.0 with heterotrophic bacteria being the predominant species present. Harrison observed the presence of ciliated and flagellated protozoa in the pool but the numbers and the species decreased as the acidity of the pool increased. Harrison was unable to isolate a Metallogenium sp. using the method of Walsh and Mitchell (1972a).

The claimed role for Metallogenium spp. has been discussed in greater detail in a previous section. Harrison (1978) suggested that organic acids produced by heterotrophic organisms, might be responsible for the sharp reduction in pH to the range that T. ferrooxidans will grow and dominate. Certain thiobacilli, e.g. T. neopolitanus, will oxidize sulphur compounds at pH values close to neutrality with the subsequent production of sulphuric acid.

6.3 Protozoa

At this time, the most direct evidence for active predation of T. ferrooxidans has involved flagellated and ciliated protozoa. These types of organisms have been identified as reducing significantly the numbers of T. ferrooxidans in both liquid cultures and a heap leaching operation with coal (McCready, 1984).

An unidentified zooflagellate was originally observed in a 20L culture of T. ferrooxidans and was responsible for reducing the bacterial cell count from 10^8/mL to 10^2/mL within an 18 hour period at pH 2.3. The predator died out rapidly and attempts to re-isolate the zooflagellate have been unsuccessful.

Flagellated protozoa have long been known to occur in acid mine waters. Ehrlich (1963) isolated a flagellated protozoa by enrichment culture from a copper mine in the Southwestern United States. The organism was co-cultured with iron-oxidizing bacteria in 9K medium (Silverman and Lundgren, 1959). In subculturing tests, the presence of the flagellate was maintained but there was always a lag phase (2 weeks) before the predator was detectable. The pH at that time was 2.0 and the flagellate appeared to feed on the T. ferrooxidans whose population had peaked after 3-4 days. There was no evidence that the flagellate could eliminate the bacterial population.

Seed (1971) was not optimistic regarding the use of ciliated and flagellated protozoa as biological control agents for T. ferrooxidans. They have, however, many attractive features.

It is generally believed that protozoa contribute materially to the decline of bacterial numbers as polluted waters are purified by natural systems (Alexander, 1971). They are highly motile and appear to respond rapidly to favourable feed densities.

Taylor and Berger (1976) noted that in a pond ecology, cohabiting ciliated protozoa were selective as to the bacteria upon which they could grow. Thus,although all laboratory strains of bacteria used in the study supported the growth of ciliates, the bacterial strains isolated from the pond appeared to be able to "discourage predation" and would not support the growth of the ciliates. This observation is important for the use of ciliated or flagellated protozoa as biological control agents. Care will be required that ciliates are isolated which are active against the wild strains of acid-generating bacteria. This may complicate the cultivation and production of larger quantities of the protozoan species. For example, will the ability to prey on wild T. ferrooxidans be lost if a bacterial strain mutates during cultivation? Another question might be whether or not a particular ciliate may discriminate in its prey selection between different wild strains of T. ferrooxidans. A ciliate effective in one situation may be ineffective at another location.

This specificity is also important in the natural environment. Mallory et al. (1983), proposed that Tetrahymena thermophilus demonstrated a high specificity for Salmonella typhimurium and Klebsiella pneumoniae. They believed that the protozoa will eliminate the prey even when an alternative prey is present at concentrations above the threshold number for active feeding by the ciliate or when the rate of growth of the prey is less than the rate of predation.

Another attractive feature of ciliated and flagellated protozoa is their tendency to encyst when they encounter an unfavourable environment e.g. lack of food, hostile pH, dessication, anaerobiosis, overcrowding, and excretion products of bacteria and other protozoa (van Wagtendonk, 1955). The cysts are

more tolerant to toxic chemicals, acids, high temperatures and they can withstand long periods of drought (Alexander, 1961). Dried cysts of some species have been found to remain viable for longer than five years. Other studies have shown cysts to survive heat treatments of 100°C (3h) and 70°C (26h) (van Wagtendonk, 1955). When favourable conditions return, the protozoa will excyst and return to an active form. Thus, a protozoa could, in theory, nearly eliminate its prey, encyst and re-emerge when the prey density has been restored to the threshold level. If such a cycle could be established against T. ferrooxidans, the requirement of repeated applications of the control organism would be significantly reduced. The cysts would also represent an ideal form for storage and subsequent application of a predator to tailings.

6.4 Rotifers

Although Seed (1971) implied that rotifers might be more effective than protozoa as control against T. ferrooxidans, no evidence was presented to support this view.

In a lake/pond ecology, rotifers tended to be present in low numbers below the surface of a body of water but present in high numbers in the sand 3-4 metres from the water's edge. They tend to concentrate in the top 3-4 cm of the sand. Alkaline waters generally contain only a few species although they will be present in large numbers. Acidic waters, on the other hand, support many species of rotifers but the sizes of the populations will be small (Pennak, 1953).

The application of rotifers to the control of acid mine drainage poses several questions. Certain rotifers do appear to ingest T. ferrooxidans (Oliver, 1967), but this occurred in mine water in a ditch which would not simulate closely the microenvironment within tailings. Attractive features of rotifers are their ability to withstand extremes of temperature and even low moisture conditions. In the latter case, some species, of the order Bdelloides are able to endure dessication. In this event, there is a drying and shrinking of the body contents and cuticle without the formation of a true cyst. A few bdelloids do form secreted cysts when subjected to drying. In their case, a gelatinous material is secreted which quickly hardens and becomes resistant.

ORF

Dessicated rotifers may be kept dry for months, or in some cases years, provided that oxygen is present. Under normal circumstances, dried rotifers can be rehydrated to normal activity within hours (Pennak, 1953).

The above characteristics suggest that rotifers are suited for application as control agents. They have the potential for long term survival under the widely varying conditions likely to occur in a tailings pile throughout the course of the year. Unfortunately, the major stumbling block will be the predator-prey balance. There is as yet, no evidence to confirm or deny the ability of rotifers to control acid-generating bacteria such as T. ferrooxidans. The unanswered question is not the ability of rotifers to prey on this autotrophic organism, but rather whether or not the autotrophic organism can be reduced or prevented from developing to sufficient numbers to produce acidic effluents.

6.5 Caulobacter spp.

Caulobacter comprise a genus of gram-negative, unicellular stalked bacteria (Henrici and Johnson, 1935). By electron microscopy, it was confirmed that this stalk was an outgrowth of the cell (Poindexter, 1964).

It was observed that in mixed cultures where caulobacters were present, the stalked Caulobacter spp. were commonly attached to the cell walls of other microorganisms (Poindexter, 1964). This suggested that the Caulobacter were preying or were parasitic towards the other bacteria. Poindexter, however, concluded that the attachment to other cells is not a parasitic action on the part of the Caulobacter spp. She observed no adverse effect on the support cell i.e no cell lysis and the Caulobacter spp. did not show any signs of enhanced growth.

The work of Shearer et al. (1968, 1970) has been discussed in a previous section. The claimed control mechanisms or acid-generating organisms would not appear to be attributable to the Caulobacter preying on the Thiobacilli.

ORF

Caulobacter spp. are aerobic heterotrophs noted for their ability to grow in environments very low in organic nutrients. They require phosphate for growth and polysaccharides appear to be involved in the mechanism by which the stalk attaches itself to a surface. If a control mechanism could be explained for Caulobacter, it would appear to be an indirect effect, possibly due to the release of organics to which the iron-oxidizing T. ferrooxidans is very sensitive.

Caulobacter generally grow at the surfaces of water, of low organic content and at pH close to neutrality. In this respect, their presence in neutral coal mine water (Browning, 1970) is to be expected. They are widespread in nature and they exhibit a relatively unusual morphology. They are non-toxic and, although it is difficult to envisage the survival of this genus in low pH tailings, the claims by Shearer et al.(1968, 1970) and Shearer and Everson (1969) should be investigated further. In this respect, Baker and Wilshire (1971) did not deny the growth of Caulobacter spp. at pH 3.2 but found rather that they did not inhibit the acid generation. Baker and Wilshire did not confirm the identity of the plaque or their flat bed reactor and as noted earlier, Shearer et al. introduced their inoculum low down in an upward flow reactor. Therefore, the test conditions were not identical.

Based on the above information, doubt remains as to a role for Caulobacter spp. in acid prevention. We do not believe, however, that it should be totally ignored since a by-product of the Caulobacter culture may be inhibitory to biological acid generation..

6.6 Bdellovibrio spp.

Bdellovibrio spp. are bacteria which parasitize other gram-negative bacterial species. Although we did not identify any literature references relating to the activity of Bdellovibrio spp. against T. ferrooxidans the latter organism is gram negative and hence is a potential prey.

Bdellovibrio spp. are active in sewers, lakes and sewage treatment systems but of the species described in the literature, none appears promising as a biological control of acid drainage from tailings. Bdellovibrio do not survive in anaerobic environments (Fry and Staples, 1974) and are relatively sensitive to toxic chemicals (Varon and Shilo, 1981). The parasitic activity of this organism appears less effective at temperatures below 26°C and the efficiency of the destruction of prey varies significantly with the respective bacterial strain and its physiological condition. The various strains of Bdellovibrio also showed marked variations in specificity and efficiency. None of these limitations is desirable for a biological control agent.

The activity of Bdellovibrio spp. is also subject to predator-prey equilibria. This is the case with E. coli in wastewaters and it has been concluded that Bdellovibrio has only a minor role in eliminating E. coli from that environment.

At the present time, Bdellovibrio does not appear to be a promising control agent for T. ferrooxidans.

6.7 Bacteriophage

Our literature review included a search for potential phage against T. ferrooxidans. None has been identified in our current search although Shearer and Everson (1969) did investigate the possibility that an inhibitor of iron-oxidizing bacteria was a phage. However, as a control agent, a virus effective against T. ferrooxidans would have tremendous potential.

6.8 Metallogenium spp.

In the above sections, we have considered known predators of bacteria and have reviewed briefly a few specific attributes of each type of organism as a potential control agent.

Another route to control of acid-generating organisms is the interruption of the pH succession of microbial communities (Walsh & Mitchell, 1972b). Despite the lack of the confirmatory experimental studies needed to verify those claims, the succession appears to centre around the species Metallogenium. We will review briefly its characteristics as they apply to biological control routes.

A Metallogenium sp. has typically an optimum pH for growth at 4.1 but will grow over the range 3.5-5.0 (Walsh and Mitchell, 1972a, 1972b). Metallogenium will oxidize ferrous iron as an energy source. It has a peculiar morphology. It tends to occur as filaments of cells (without conventional cell bodies). The filaments break very readily upon shaking and the individual fragments tend to be very small and are not retained by a 0.45 micron filter.

Metallogenium spp. will grow in the presence of 0.4% formalin whereas T. ferrooxidans will not (Walsh and Mitchell, 1972b). This ability is useful in isolating the organisms by enrichment culture.

Walsh and Mitchell (1972b) identified a role for Metallogenium in a pH dependent succession of iron bacteria loading in a tailings pile to a dominant culture of acid-generating species (T. ferrooxidans). They proposed several routes to biochemical control of acid generation (1972c). In addition to the use of ferrous iron concentrations (e.g. 100 ppm) to inhibit the growth of Metallogenium as discussed earlier, they suggested:

1. Application of surface active agents to dislodge Metallogenium stalks from active sites.
2. Seeding or inducing growth of an organism antagonistic to Metallogenium stalks.
3. The use of a toxic agent specific for Metallogenium.

7. CONCLUSIONS

We have completed an in-depth review of the literature pertaining to the use of biological agents to control acid generation in pyrite-containing tailings. This study has included a critical assessment of not only organisms which may utilize T. ferrooxidans as prey, but also those approaches based on the inhibition of a key reaction in the succession of reactions leading to the oxidation of pyrite minerals in tailings and the release of sulphuric acid.

We have identified several methods to be examined further at the laboratory level. The development of these methods is not far enough advanced to warrant, at this time, an assessment of factors such as the effect of process parameters, economics, environmental safety and related technological parameters.

Based on available data, predatory organisms are ranked ahead of other approaches. In the literature, data on the predatory activity of rotifers and protozoa against T. ferrooxidans are restricted to a few isolated and poorly documented observations. These predators, however, have been identified as either cohabiting and preying on T. ferrooxidans in acid mine water or eliminating T. ferrooxidans from microbial leaching operations.

Rotifers and protozoa have many attractive features including their ability to withstand relatively severe environmental stresses such as dehydration, shortage of nutrients, low or high temperatures, acidity, etc. This may involve either dessication in the case of rotifers or the formation of cysts in the case of protozoa. These organisms may show a high level of specificity towards prey bacteria and can cause significant reductions in bacterial numbers over relatively short time intervals. There are, however, many factors which must be investigated at the bench.

We also identify several other approaches which warrant further studies. In general, the experimental data for these methods are incomplete and often contradictory. In some situations, such as the application of Caulobacter spp. or the inhibition of Metallogenium, the positive claims of the originators of the work were not duplicated by other laboratories. This appears to have discouraged the undertaking of additional studies. We do not consider that the evidence, as published, for either of these approaches is sufficiently conclusive to accept or reject them. We recommend that they should be investigated further at the laboratory level in order to obtain more definitive data.

These methods (i.e. the use of Caulobacter or inhibition of Metallogenium) would benefit from limited studies at a more basic level. They represent unique strategies for control of acid generation which, although not validated to-date by other laboratories, may lead to practical technologies.

On the basis of current evidence we do not regard Caulobacter spp. as predators or parasites. The data on this organism as published by Shearer et al. (1968, 1969, 1970) are confusing. The claimed inhibition may not have been due to Caulobacter, which would explain the contradictory evidence from Baker and Wilshire (1971).

Similarly, a role has yet to be confirmed for Metallogenium spp. in the succession of iron-oxidizing bacteria leading to acid generation in tailings etc. It is, however, one explanation for the mechanism by which the pH in the tailings can be reduced more rapidly to the value that favours growth of T. ferrooxidans.

The feasibility of using other predatory organisms, such as Bdellovibrio spp. or viruses has not been indicated by any data in the literature.

8. RECOMMENDATIONS FOR FUTURE STUDY

McCready (1984) has identified a flagellated ciliate in a coal heap-leach operation. This organism is depleting the T. ferrooxidans cells and would be ideal for immediate investigations. The initial laboratory tests would involve studies to evaluate the performance of the ciliate against T. ferrooxidans in column reactors of both tailings and coal.

Studies should also include a comparison of methods to isolate and maintain viable cultures of the ciliate.

As part of a continuing program, we recommend that samples of tailings and acid drainage from the Nordic Tailings (Elliot Lake) should be obtained. They will be examined for the presence and isolation of possible protozoan predators of T.ferrooxidans. Acid mine drainage from other locations should also be investigated.

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APPENDIX I

APPENDIX I

COMPUTER-ASSISTED LITERATURE SEARCH FOR PREDATORS - KEYWORDS

Caulobacter
Bdellovibrio
Rotifer
Review
Prey
Predator
Parasite
Biological Control
Phage
Bacteriophage
Thiobacillus
Iron-oxidizing Bacteria
Leaching
Tailings
Acid
Mine
Inhibit
Virus
Protozoa
Ciliates
Interaction
Metallogenium

APPENDIX II

APPENDIX II

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BIOLOGICAL CONTROL OF THIOBACILLUS FERROOXIDANS BY PREDATORY ORGANISMS

PHASE II

ORE

1. OBJECTIVE

In Phase II of the study, the objective was to isolate and culture organisms predatory to T. ferrooxidans and to assess the feasibility of using such cultures to inhibit and/or control the microbiological generation of acid in mine tailings.

2. EXPERIMENTAL STRATEGY

The potential predatory organisms identified in our literature search included bacteria (Caulobacter spp.), rotifers and protozoa. Information on the abilities of the different types of organisms to control the number of T. ferrooxidans within tailings was both very limited and inconclusive.

However, Dr. R.G.L. McCready, Dalhousie University observed that a ciliated contaminant introduced inadvertantly into a coal heap leach process using T. ferrooxidans, reduced significantly the numbers of T. ferrooxidans cells (by several orders of magnitude). Such evidence suggested that the use of rotifers or protozoa be investigated. This was recommended in the Phase I report. It was also recommended that water and sediment samples collected from the vicinity of acid-generating tailings at the Nordic site, Elliot Lake be investigated as sources of predatory organisms.

An important aspect to be considered in the design of an experimental program was the method by which such free-swimming predatory protozoa might be used to control acid generation in the field. Two control scenarios might be envisaged. The first situation would involve the application of predators to tailings before acid-generating species have become established. The second approach would apply where biologically-catalyzed acid generation was already occurring within the tailings. In the former case a pH range of 5 - 8 might be expected within the tailings. In the latter case, the predator would have to be effective in a much more acidic environment, i.e., as low as pH 2.0.

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A further complication is that due to anthropogenic causes, the prevailing pH of rainfall at many mine sites may already be close to 4.0. Thus, the leaching effect of the rainfall will favour the creation of a more acidic environment within the tailings. It was concluded, therefore, that predatory species must be able to survive at lower pH values (less than 4.0).

A final consideration would be the ability of predators to penetrate and survive in lower zones of tailings heaps. Thus, factors such as physical size, mobility and sensitivity to oxygen depletion must be borne in mind.

3. EXPERIMENTAL PROGRAM

3.1 Isolation and Culturing of Predator Organisms

3.1.1 Organisms isolated at the Laboratories

Following his initial observation, Dr. R.G.L. McCready was unable to isolate the extremely effective "killer" organism noted above. In subsequent tests he isolated other types of predatory species (flagellated and ciliated protozoa) but major difficulty was experienced in transporting samples of these cultures to our laboratory. Thus, cultures containing Escherichia coli as prey were forwarded by courier, collected in person from Halifax by Dr. J. Christison or delivered in person to the Ontario Research Foundation by Dr. McCready. Except for one sample, these cultures did not survive the inter-laboratory transport. In all instances, the samples examined microscopically were devoid of active protozoa upon arrival at the Ontario Research Foundation. The addition of T. ferrooxidans or E. coli cells did not restore actively growing cultures. Thus, if encystment had occurred the reversal (excystment) was not being achieved. The decision was made to isolate active predators in our laboratory.

3.1.2 Organisms Isolated at Ontario Research

Throughout these investigations, T. ferrooxidans cells were used as prey. Their use appeared to afford more consistent results than was found in limited tests with E. coli as prey. Since the majority of the predatory cultures were mixed (i.e., not pure cultures of single organisms), the use of T. ferrooxidans would reduce the risk of isolating organisms unable to use this bacterium as prey. It was noted in our literature search that protozoa can exhibit species-specific and even strain-specific preferences for their prey (Taylor and Berger, 1976; Mallory et al., 1983).

However, the use of T. ferrooxidans required labour intensive preparation procedures. T. ferrooxidans cells were recovered by centifugation from liquid cultures in 9K medium (Silverman and Lundgren, 1959). The cells were separated from iron precipitates by suspending the combined residues in sulphuric acid solution (pH 1.5) followed by low-speed centrifugation to separate the heavier iron particulates from the cells (Silverman and Lundgren, 1959). The cells in turn were recovered by centrifugation at higher speed and washed with sterile deionized distilled water until the pH of the suspension in water was greater than 3.5.

3.1.2.1 Samples From Elliot Lake

Following the spring thaw, a group of ten (10) liquid and five (5) sediment samples were collected in mid-May, 1984 at the Nordic Tailings, Elliot Lake. The pH values of the water samples were measured. Aliquots(5 mL of water samples and approximately 0.1 mL of solid samples/suspensions) were inoculated into 9K medium (100 mL) and incubated with shaking to identify the presence of T. ferrooxidans in the natural samples.

To test for the presence of predators, an aliquot (9 mL of liquid samples and approximately 0.1 mL of the solids/suspensions) was added to 1 mL of a concentrated suspension of T. ferrooxidans cells. Where necessary, sterile deionized water was added to dilute the suspension to 10 mL. To each sample, 9K medium (1 mL) was added. The cultures were incubated without shaking at room temperature. Several of the Elliot Lake samples (particularly the slurries) had significant algal populations. Several chlorophyll-containing organisms were dominant in some cultures and eventually the test mixtures were incubated in the dark to inhibit the growth of photosynthetic species.

The cultures in the presence of added T. ferrooxidans cells were monitored at regular intervals. Motile and non-motile protozoa were noted in several samples and successive sub-culturing of these cultures were identified by two tests (both microscopic) namely a decrease in the T. ferrooxidans population compared with a control culture and the visible presence and increase in numbers of protozoa, particularly those of the motile variety. Direct counting of the cells was not feasible since the cells in some instances tended to flocculate. In other cultures, active iron-oxidation was noted. This would lead to loss of cells from free suspension by either adsorption on to glass surfaces or to particulate iron compounds. Throughout these isolation tests, the goal was to identify major changes in cell or predator numbers.

A promising isolate was obtained from one sample (3A). Subsequently, further isolations were carried out from this Elliot Lake sample during the following months. These later isolation experiments used T. ferrooxidans suspension added to an aliquot of 3A and diluted with sterile distilled water. Dilution had little effect on the natural pH of the sample (pH 3.2). Subsequent transfers were made into T. ferrooxidans cells in aqueous suspension pH 3.0 - 3.5. An isolate of interest, culture 3A, resulted from these experiments. At a later date, a second enrichment culture was obtained from the original slurry of sample 3A. This second culture was identified as culture 3A-2.

3.1.2.2 Samples From Dr. R.G.L. McCready

Two liquid samples were received from Dr. R.G.L. McCready, Dalhousie University. The first sample contained a ciliate population surviving on E. coli as prey (cells had been washed with 0.15 M phosphate buffer, pH 7.0). The second sample was a pond water (Sullivan's Pond). Both samples appeared to have viable protozoa which had survived the shipment. These samples were incubated with T. ferrooxidans cells at both pH 2.5 (dilute 9K' medium) and at pH 5.5 (dilute aqueous suspension). Predator cultures were isolated and maintained on T. ferrooxidans as prey. One of these cultures, JC-3 was investigated in greater detail.

3.1.2.3 Samples From Local Sites

As part of the on-going search for improved cultures of predators, approximately 20 samples of water were collected from ditches, ponds and run-off from fields in the Peel/Halton Regions of Ontario. Enrichment cultures used T. ferrooxidans suspension adjusted where so desired to pH 3.0 or 5.0.

3.1.2.4 Alternative Culture Techniques

As indicated earlier, the use of T. ferrooxidans was regarded as important during the enrichment culture of a suitable predatory organism(s). However, the production and preparation of cleaned T. ferrooxidans cell suspensions was time-consuming and expensive. Furthermore, when tests of the effects of predator cultures on the acid-generating ability of tailing specimens were to be carried out, it would be desirable if a culture method could be used which did not use T. ferrooxidans or other cells as prey. Thus active T. ferrooxidans cells would not be introduced with the predator. A range of dilute organic media was selected from text books on the culture of protozoa (Manwell, 1961; Kudo, 1977). The media included Media N and P for ciliates (Hall, 1937), diluted hay infusate, Media G for colourless flagellates (Hall, 1937), and rice/yeast medium (Jackson and Berger, 1984) (see Appendix I)

3.1.2.5 pH Stability of Test Cultures

Two more promising cultures were selected for further study: culture JC-3, a flagellate in relatively pure culture growing well at pH 5.5 and culture 3A-2 (from Elliot Lake sample), a mixed culture growing very actively at pH 3.0 - 3.5.

Culture JC-3 was more sensitive to low pH values. Acetate buffer (0.001 M) was used to adjust the pH of T. ferrooxidans/predator cultures to 3.6, 4.0, 4.5 and 5.0, respectively. Tests had shown that dilute acetate ion did not inhibit the growth of the predator at pH 5.5. Culture 3A-2 was tested by adjusting the pH of T. ferrooxidans suspensions with dilute sulphuric acid solution to the lower pH value. The utilization of T. ferrooxidans was monitored by measuring the absorbance at 600 mμ.

3.1.2.6 Tests of the Ability of Culture 3A-2 to Utilize T. ferrooxidans

In all of the isolation tests, washed cell suspensions of T. ferrooxidans were used with no other organic nutrients being deliberately added. An additional test monitored both the absorbance of the suspension at 600 mμ and the estimation of the T. ferrooxidans population using a serial dilution culture test (4 dilutions) in 9K medium.

3.1.2.7 Photographic Techniques

Cultures of JC-3 and 3A-2 were examined by both transmission and scanning electron microscopy. In the former method, the cultures were fixed with 1% gluteraldehyde and negatively stained with 2% phosphotungstic acid. In the latter procedure, the culture samples were fixed in gluteraldehyde, post-fixed in with osmium tetroxide, dehydrated in alcohol and critical point dried in carbon dioxide.

3.2 Tests with Tailings Samples

3.2.1 Tailings Received From Falconbridge

Samples of neutralized fresh tailings were received from Falconbridge. The sample was acidic and required extensive washing with distilled water to raise the pH value.

A suspension of the Falconbridge sample was prepared. Predator culture (JC-3 or 3A-2) was added to the suspension which was then analyzed under the microscope. The particle size of the tailings was extremely small, i.e., generally of the same size as the predators. It was obvious that this particle size would prevent adequate access of the predators into a tailings pile. The particle size also caused severe blockage of columns being prepared for leach tests. The tailings did not appear to be toxic to the predators. A sample of the Falconbridge tailings was dried and analyzed for its particle size distribution.

3.2.2 Tailings Received From Inco Ltd., Sudbury - Lysimeter Test 1

These pyrrhotite tailings were much coarser than the Falconbridge sample. Six lysimeter boxes (8.5" long x 6" wide x 12" deep) were constructed out of standard plastic containers. A false bottom was created in each container from 100 mesh synthetic screen cloth which acted as a filter. Each container was placed in a larger plastic container (13" x 7" x 15") installed with a draw spout at the bottom. A cushion of fine silica sand was provided between the two containers. The outer container was raised at the rear to

give a 30° angle for drainage purposes. The net weight of tailings in each lysimeter box was:

Box No. 1	7.683 Kg
2	7.500 Kg
3	7.457 Kg
4	7.799 Kg
5	7.799 Kg
6	7.500 Kg

The pyrrhotite tailings as received were acidic (pH of the discharge being 2.2-2.4). The tailings in the lysimeters were wetted daily with sodium hydroxide solution pH 11-12 and washed continuously with distilled water. This procedure was repeated for several days until the pH of the eluant collected from the bottom of each lysimeter was higher than 4.1. When the desired pH was attained in all lysimeter boxes, inocula of predators were prepared.

A 50 mL culture of predator 3A-3 was cultivated on T. ferrooxidans as prey in aqueous suspension pH 3.3. When ready for inoculation, the culture was divided into two equal volumes. One sample was diluted with sterile distilled water to 100 mL (Active Predator/T. ferrooxidans inoculum). The other aliquot was adjusted to pH 1.75 with sulphuric acid and heated to 55°C for 5 minutes. This treatment inactivated the predators present. The mixture was cooled, the pH adjusted to 3.3 with sodium hydroxide solution, and the whole diluted to 100 mL (Deactivated Predator/T. ferrooxidans inoculum).

The respective lysimeters were inoculated with predator 3A-2 and T. ferrooxidans according to the following scheme:

Box 1. - No inoculum. Percolating solution, distilled water pH 4.1.

2. - Inoculated with 50 mL T. ferrooxidans/deactivated predator solution. Percolating solution, distilled water pH 4.1.

3. - Inoculated with 50 mL T. ferrooxidans/deactivated predator solution. Percolating solution, tap water pH 4.1.

4. - Inoculated with 50 mL T. ferrooxidans/active predators. Percolating solution, tap water pH 4.1.

5. - Inoculated with 50 mL T. ferrooxidans/active predators. Percolating solution, distilled water pH 4.1.

6. - No inoculum. Percolating solution tap water, pH 4.1.

The lysimeter boxes were inoculated with the respective culture using a pipette to evenly disperse the solution across the surface of the

tailings. The solution was washed in with a small volume of distilled water or tap water respectively.

The volume of liquid in each lysimeter was maintained at a set level. Each day a known volume of seepage or drainage was drained from each lysimeter box and replaced with an equivalent volume of the appropriate percolator solution. The pH of each discharge was measured. These discharge solutions were also examined regularly for the presence of predator species.

3.2.3 Inco Tailings - Lysimeter Test 2

The lysimeter boxes + tailings used in the previous test were once again treated with alkali and water to gradually restore the pH of the discharge from each tailings column to greater than 4.1.

When all lysimeter discharges were greater than 4.1, the boxes 2, 3, 4, and 5, were inoculated with active and inactive predator cultures as described in the previous test. The experiment was conducted as described before with the pH of each discharge being monitored. At selected intervals, 15 tube (3 dilutions x 5 tubes) MPN estimates of T. ferrooxidans numbers were performed on the discharges. The lysimeter boxes were reinoculated with predator 3A-2 and inactivated control cultures at regular intervals, i.e., Day 0, Day 3, Day 6, Day 10, Day 13, and Day 15. At selected intervals, samples were taken from the liquid within each lysimeter box and this sample was examined under a microscope for the presence of predator organisms. The discharges were also examined for the presence of protozoa.

4. RESULTS AND DISCUSSION

4.1 Isolation of Organisms

4.1.1 Tailings Samples From the Nordic Tailings, Elliot Lake

The description of the sampling sites at the Nordic tailings is given in Table 1 and shown in Figure 1. The pH values of the water samples

were generally in the range of 2.73 - 3.09 (Table 2). One sample (No. 8) taken from a swamp area at the fringe of a lake where there was no tailings had a higher pH value (3.90). Except for sample No. 1A (pH 4.73), the solid suspensions (approximately 1.00g in 25 mL distilled water) all gave strongly acidic solutions.

Aside from sample No. 8, all of the water and sediment samples gave positive tests for T. ferrooxidans when cultured in 9K medium (15 days' incubation). Thus, any predators indigenous to these samples were not preventing the development of T. ferrooxidans populations. This is supported by both the pH values and the microbial culture tests. These cultures were left for 30 days but no protozoan predators were detected in them.

When an aliquot of each Elliot Lake sample was incubated with suspensions of T. ferrooxidans in dilute (1:10) 9K medium, iron-oxidizing activity was not inhibited. The cultures were examined at regular intervals. Algal species were predominant in several samples, most noticeably in the solid samples. In subsequent tests, cultures were maintained in the dark to inhibit the growth of these contaminants. These algae would not be viable at lower unilluminated zones of tailings. Their presence,however, reflects the samples being taken from the surface zones of the tailings.

In the test culture for 3A, it was noted after a period of one month that the T. ferrooxidans population had been significantly reduced (compared with the other cultures and a control). Accordingly, this sample was subcultured into fresh T. ferrooxidans suspensions and a motile flagellated protozoa was obtained (Culture 3A). However, difficulty was experienced in maintaining this culture. Following a relatively slow rate of growth, the numbers of the active predator would peak rapidly. This would be

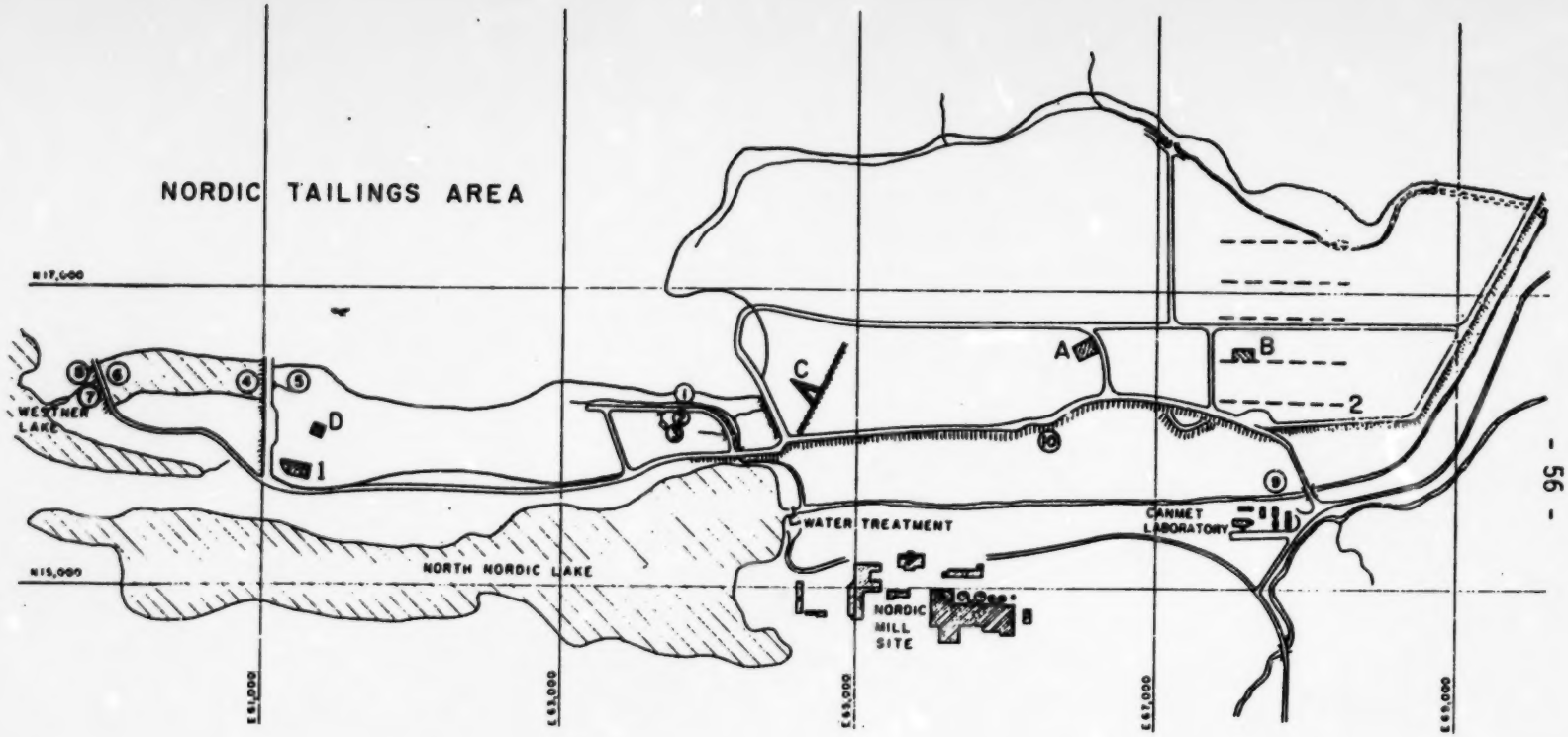

Figure I. SAMPLING SITES AT NORDIC TAILINGS, ELLIOT LAKE.
(For description see Table I)

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TABLE I: LOCATION OF SAMPLING SITES AT THE NORDIC TAILINGS (WEST ARM) ELLIOT LAKE, ONTARIO - MAY 15, 1984

1.	Shallow stagnant water marsh land in the creek above tailings, bulrushes.
2.	Shallow stagnant water pooled in tailings, bulrushes.
2A.	Sludge above water at site No. 2
3.	South side of water pool as of No. 2, tufts of grass.
3A.	Sludge above water site No. 3.
4.	Holdings pond east end, deeper water.
5.	Stagnant water pool near holding pond, shallow water, algae.
6.	Holding pond west end, deeper water.
7.	Seepage water from holding pond dam.
8.	Stagnant water above Wesner Lake swamp, no tailings.
9.	Water from drainage ditch (murky water).
10.	Stagnant water, seepage from Main Nordic Dam, algae.
10A.	Beach sludge sample site No. 10.

TABLE II: EVALUATION OF WATER AND TAILINGS SAMPLES FROM NORDIC TAILINGS, ELLIOT LAKE

SAMPLE NO.	DESCRIPTION	pH	TEST FOR T. FERROOXIDANS ①				PRESENCE OF PROTOZOA IN T. FERROOXIDANS SUSPENSIONS (Day 20) ④
			INOCULUM	DAY 7	DAY 9	DAY 15	
1	Liquid	2.92	5 mL	- ③	+ ③	+	Yes
2	Liquid	3.09	5 mL	-	+	+	Yes
3	Liquid	3.08	5 mL	-	+	+	Yes
4	Liquid	3.05	5 mL	-	-	+	No
5	Liquid	3.05	5 mL	-	+	+	No
6	Liquid	3.04	5 mL	-	-	+	Yes
7	Liquid	3.15	5 mL	-	-	+	No
8	Liquid	3.90	5 mL	-	-	-	No
9	Liquid	2.99	5 mL	+	+	+	No
10	Liquid	2.73	5 mL	-	+	+	No
1A	Slurry	4.73 ②	.1 g	+	+	+	No
2A	Slurry	3.02 ②	.1 g	+	+	+	Yes
3A	Slurry	3.00 ②	.1 g	+	+	+	Yes
9A	Slurry	2.59 ②	.1 g	+	+	+	No
10A	Suspension	2.60	5 mL	+	+	+	Yes

① 9K medium, 29°C

② 1.00 g suspended in 25 mL distilled water

③ + = iron oxidation detected ; - = no iron oxidation detected

④ Test samples incubated with T. ferrooxidans cells in dilute 9K medium

followed by a rapid decrease in viable numbers although no cyst formation could be detected by microscopic examination. This culture was lost. It was noted that at the time of "death" the T. ferrooxidans population was still signficant, e.g., estimated to be greater than 10^4 cells per mL of suspension. Thus, the predator was not achieving an elimination of the T. ferrooxidans population.

Following these observations, a second culture was isolated from sample 3A. This culture, 3A-2 was selected for further study.

4.1.2 Samples From Dr. R.G.L. McCready

As noted earlier, difficulty was experienced in transporting samples of ciliated protozoa from Dr. McCready's laboratory to Ontario Research. These cultures appeared very sensitive to environmental conditions. From one sample of pond water forwarded to us by Dr. McCready, three cultures were obtained by incubating them with T. ferrooxidans at pH values greater than 5.0. In one of these cultures, the predominant organisms varied with the age of the culture and eventually large protozoa (greater than 70 microns in length) tended to dominate. These organisms were voracious and difficult to maintain due to their large consumption of cells.

The large size of the organisms was considered to preclude their use in a tailings environment and therefore they were discarded. However, one culture, predominantly a smaller biflagellate, appeared to grow well in the presence of T. ferrooxidans and to suit our requirements. This culture, JC-3, was selected for further study.

An interesting addendum to these observations was that the cultures isolated from these pond samples by Dr. McCready and ourselves did not resemble each other (McCready, 1984). It is possible, therefore, that the transporting of the pond water may have caused some changes in the make-up of the protozoan population. These variations may also have been due to differences in the enrichment protocols used in the respective laboratories.

4.1.3 Samples From Peel/Halton Regions (Ontario)

Approximately twenty different samples of ditch water, ponds, run off from fields, etc. were tested. None of the samples afforded predators to T. ferrooxidans active at the lower pH range (2.5-3.0). However, several yielded very active protozoan populations predatory towards T. ferrooxidans at the higher pH values (5 - 7). In each case, the larger types of organisms, e.g., ciliates greater than 50 microns in length or diameter were predominant. Such organisms were not considered suitable for the intended application in fine particulate tailings. None of these cultures appeared better than the cultures described above and hence they were discarded.

4.2 Examination of the Test Cultures

4.2.1 Predatory Behaviour

The cultures 3A-2 and JC-3 described earlier were evaluated. The ability of each culture to reduce the numbers of T. ferrooxidans in cultures where this microbe was provided as the sole nutrient, was demonstrated. Based on a decrease in the optical density of the suspensions combined with visual estimation by microscope examination, both cultures multiplied in the presence of this bacterium as prey and were active predators. As noted in Figure 2, JC-3 grew well at pH 6.2 but had no action at pH 3.6 (It survived well at pH values as 4.5). Tests of the effect of pH on JC-3 used dilute sodium acetate/acetic acid buffer(0.001M) to control the pH values at 4.5 and 5.0. At pH 5.5, 0.001M sodium acetate was not toxic to the growth of the protozoa. However, the addition of acetic acid to adjust the pH values may have introduced a toxic component. It was observed that the T. ferrooxidans cells clumped heavily at the lower pH values in the presence of the acetate buffer.

The mixed predator culture, 3A-2, grew well at pH 2.5-3.5. Following a lag period of several days, there was a sharp decrease in the absorbance of the test suspensions. Based on the decrease in the absorbance of the cultures (Figure 3) the predatory activity was slightly better at pH 3.0 than at 3.5. In these tests, sulphuric acid was used to adjust the suspensions to the desired pH values. The growth at pH 2.5 was much slower and was not monitored. In a separate test (Figure 4), a similar decrease in the absorbance of the bacterial suspension was noted at pH 3.2 when inoculated

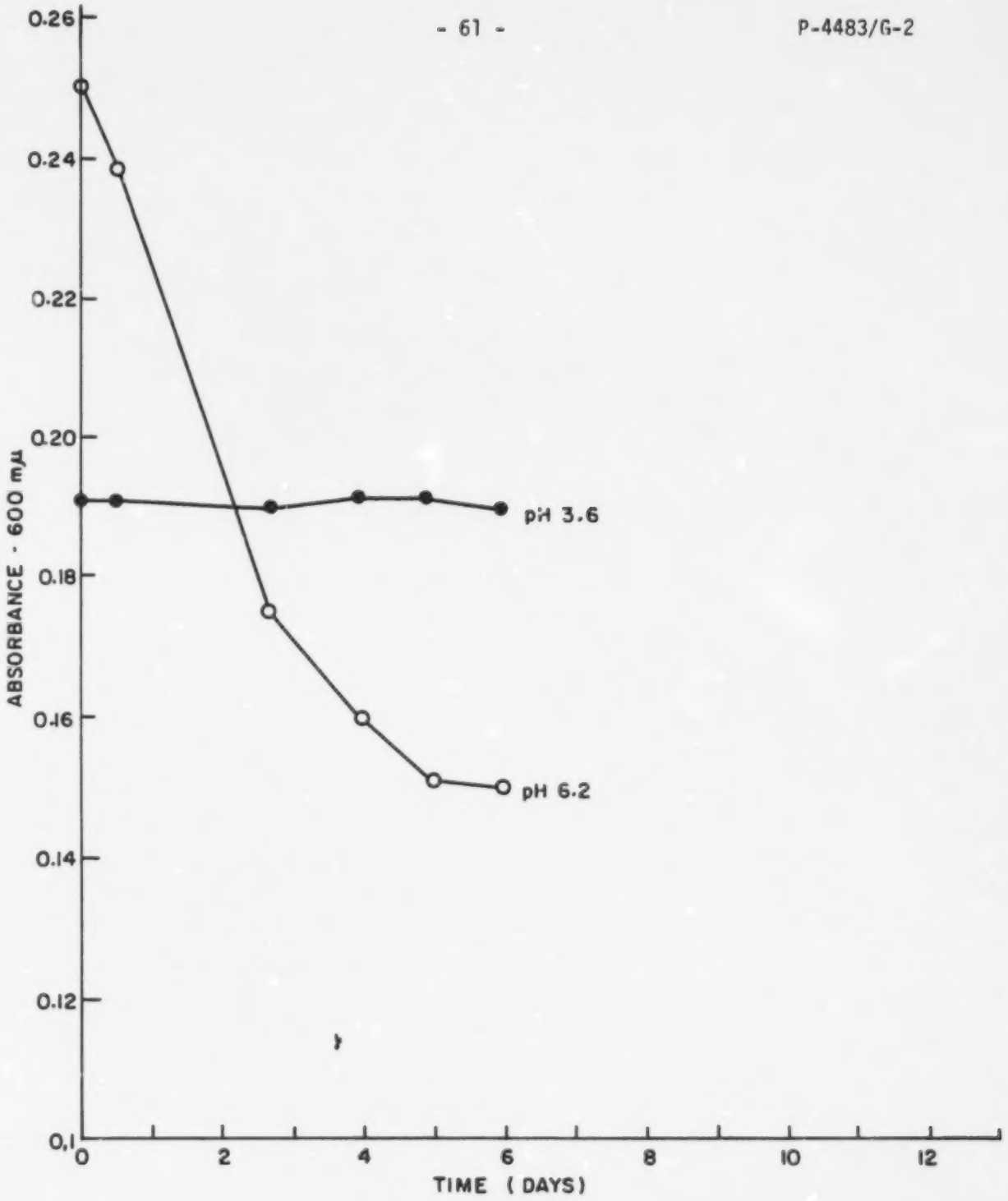


FIG. 2 UTILIZATION OF THIOBACILLUS FERROOXIDANS BY PREDATOR 3A-2
EFFECT OF pH

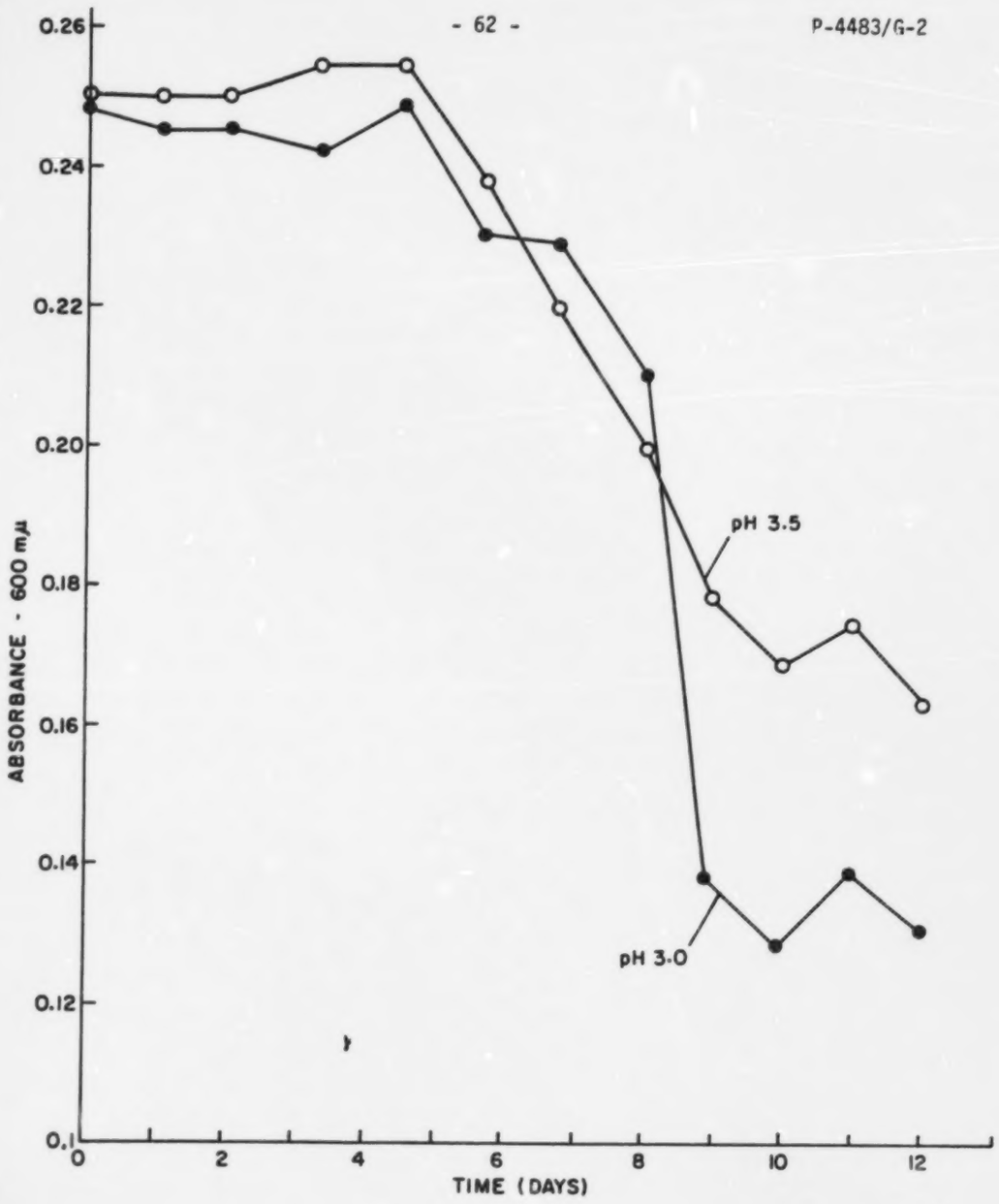


FIG. 3 UTILIZATION OF THIOBACILLUS FERROOXIDANS BY PREDATOR 3A-2
EFFECT OF pH

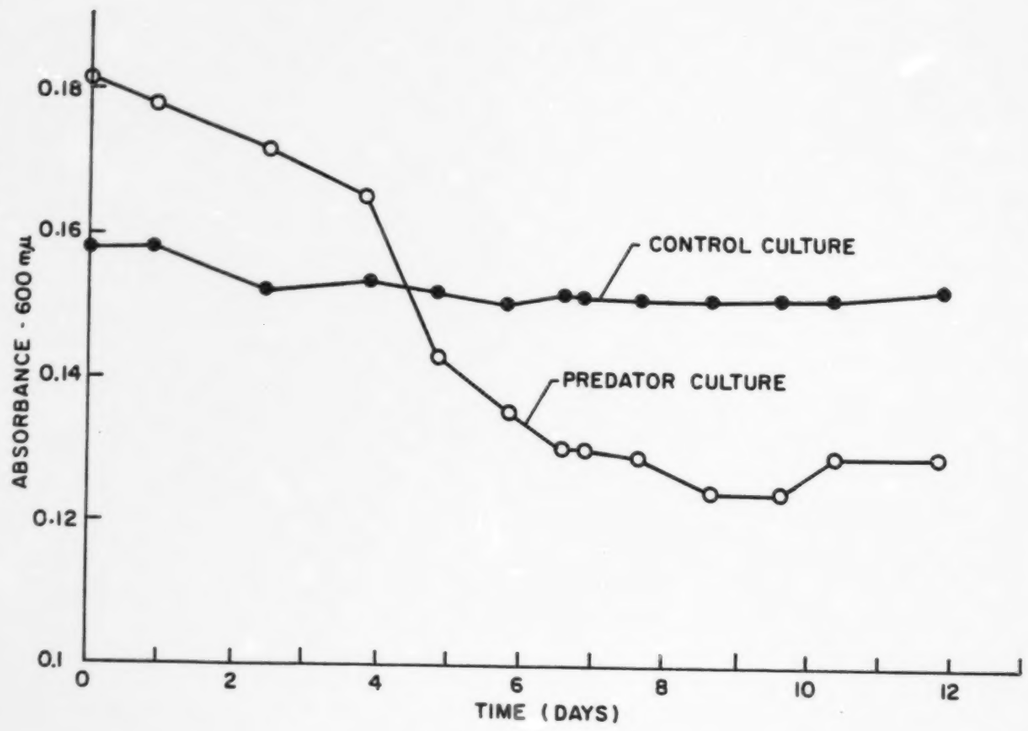


FIG. 4 UTILIZATION OF THIOBACILLUS FERROOXIDANS BY PREDATOR 3A-2
pH 3.2

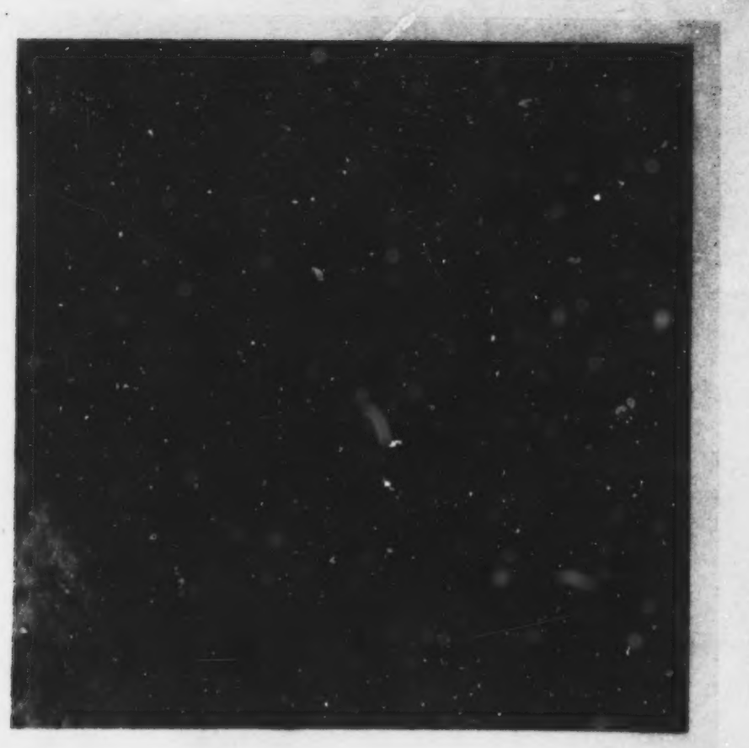


Figure 15: Protozoan forms in predator culture JC-3. Magnification x 39,000. Transmission electron microscopy.

The photographs show the relative sizes of the protozoa and the bacteria. The T. ferrooxidans are typically short rods. The bacteria in Figure 5 are estimated to be approximately 0.5 x 1.7 microns. Figure 9 shows the bacteria closely associated with the protozoon - possibly in the process of being digested.

Most of these organisms in liquid culture moved with a circular/gyratory motion. Certain pear-shaped organisms rotated lateral to the direction of movement, and moved similar to a torpedo.

4.3 Alternative Culture Techniques

Throughout this preliminary study acid-washed T ferrooxidans was used as the prey for the isolation and maintenance of the predator cultures. The production of the necessary T. ferrooxidans cells was a very labour intensive operation. Escherichia coli was investigated at the outset as a prey for culture JC-3. The culture did appear to utilize the E. coli but growth was much slower than in the case of the T. ferrooxidans supported cultures. There also appeared to be a change in the predominant organisms with the different preys. The E. coli cells are typically larger, i.e. 1.1-1.5 x 2.0-6.0 microns, than the T. ferrooxidans at 0.5 x 1.0 microns (Buchanan and Gibbons, 1974) and this may have contributed to differential enrichment of predators in response to the respective species of prey.

Alternative culture media based on dilute organic nutrients failed to support the growth of culture JC-3. Positive results appeared to be obtained with diluted hay infusate but subsequently it was apparent that the survival of the JC-3 organisms was more probably due to their utilizing the heterotrophic bacterial contaminants whose growth requirements were met by the hay infusate. These heterotrophs were present at all times in the predator cultures. The failure to obtain positive survival of JC-3 in the various organic/salts media led us to reject this approach at the present time. Culture 3A-2, isolated at a later stage in the program, was not tested with these alternative media.

with predator culture 3A-2. No change occurred in the control (uninoculated) culture over the incubation period of 12 days. The original absorbance of the test culture was lower in this test indicating a lower initial bacterial population. As a result, the growth of the predator and the concomitant decrease in the absorbance was less significant than in Figure 3 which commenced with a higher bacterial population.

During the test shown in Figure 4, samples of both the control and test cultures were removed at selected intervals and serially diluted into 9K medium to estimate the bacterial cell numbers. Dilutions were selected with the anticipation of observing major changes (i.e. several orders of magnitude) in the bacterial population of the test culture. However, based on the MPN calculations from standard tables, all cultures exceeded 10^4 cells/mL using a 10 day incubation period at 20°C. The times taken for the highest dilution (x 10^3) of the test culture samples to show a strong positive iron-oxidizing reaction differed over the course of the experiment. These times were used to arrive at estimates of the respective cell populations. Assuming that a T. ferrooxidans population of 10^7cells/mL is required to give the above positive reaction in a dilution test and using the conservative estimate of 13h for the mean generation time, the data indicated that the T. ferrooxidans population decreased by only a single log unit (10^7 to 10^6 cells/mL) between the start of the test and the twelfth day. Thus, while the predatory organisms were confirmed to utilize the T. ferrooxidans as prey, they did not appear able to reduce the bacterial numbers below approximately 10^6cells/mL or alternatively the predators required this minimum number of cells for vegetative growth. This was noted with both the JC-3 and the 3A-2 cultures. Bacterial counts estimated using a Petroff-Hausser cell counter gave a similar pattern of results although the values tended to be higher (e.g. 10^9 cells/mL) with this method.

Whichever counting method was used, a potential source of error was the tendency of the T. ferrooxidans cells to attach to particulate contaminants or alternatively to "swarm" (Figure 5). This clumping tendency appeared to be mediated by the metabolic activities of the predator organisms

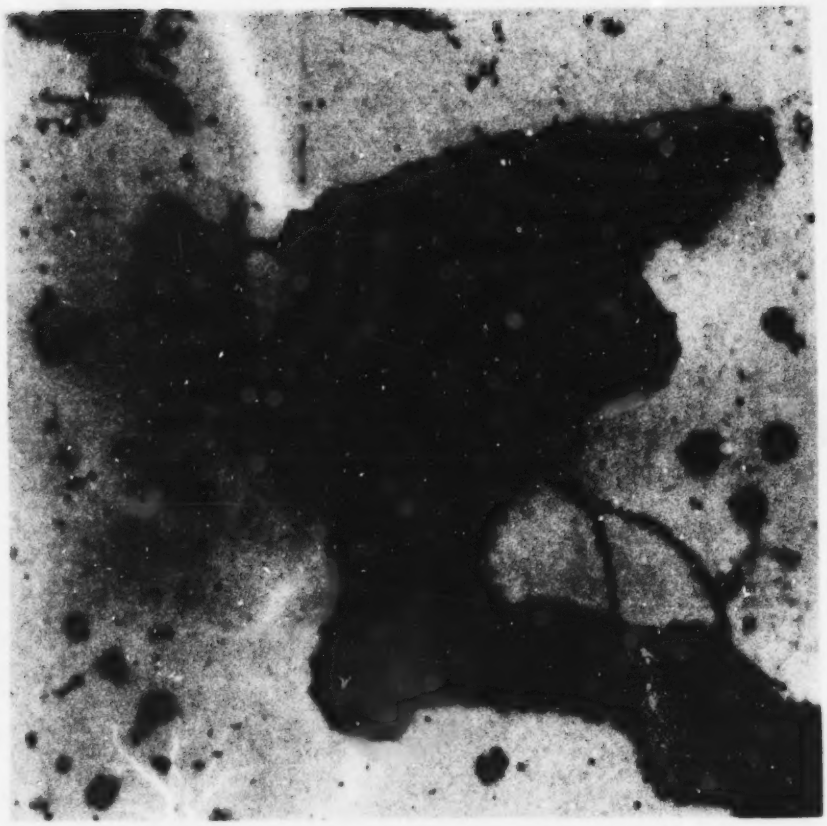


Figure 5: Swarming bacterial cells from predator culture 3A-2. Magnification x 21,000. Transmission electron microscopy.

and in some instances, the clumps would consist of several hundreds of bacterial cells. It was generally more prevalent at the later stages of the incubation of cell suspensions with predators but in some instances it occurred early in the incubation period.

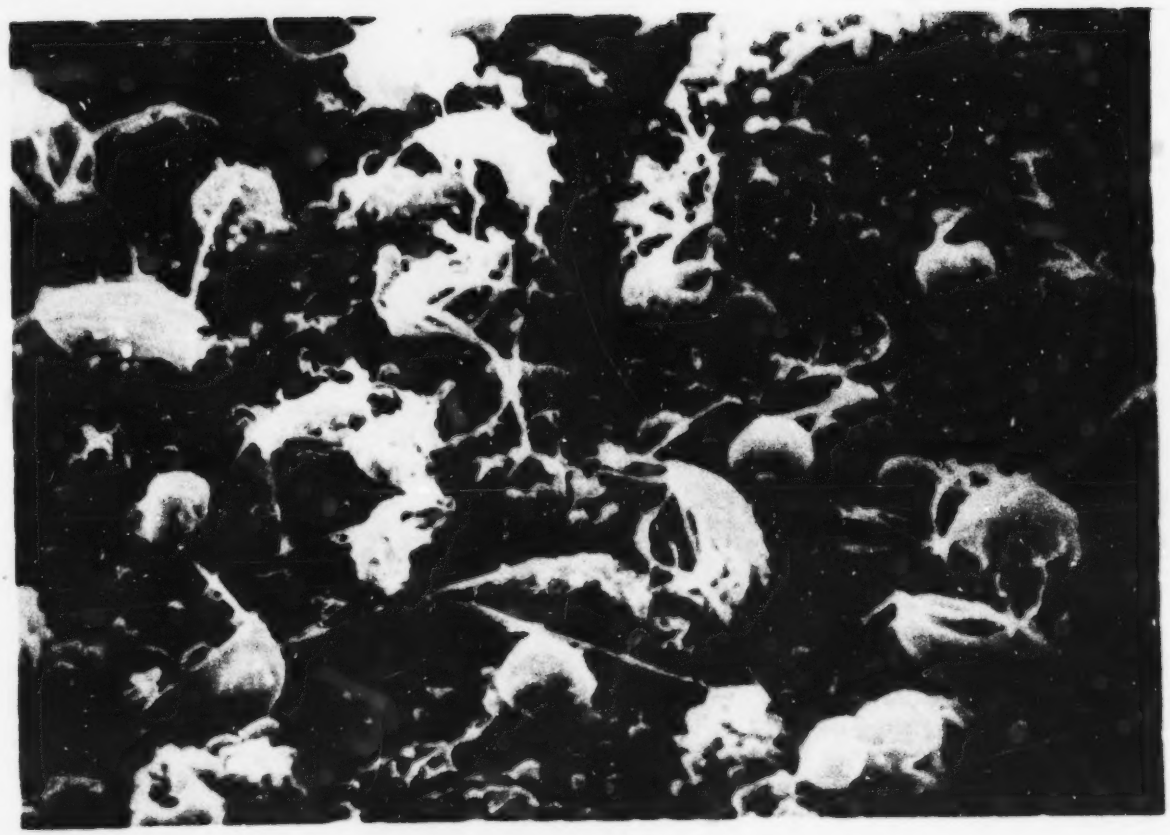
4.2.2 Morphology of Protozoa

Figures 6 - 8 show the appearance of some of the protozoa recovered from active cultures of predator 3A-2. The photographs were obtained by scanning electron microscopy of organisms which had been removed by filtration from cultures and subjected to critical point-drying. In all photographs, the flagellated protozoa rest on a "mattress" of bacterial cells. Scanning electron micrographs of culture JC-3 showed no identifiable protozoan forms.

Figures 9 - 13 are examples of protozoa from culture 3A-2 prepared by negative staining with photographs by transmission electron microscopy. The darkening zones indicate where cell lysis was occurring.

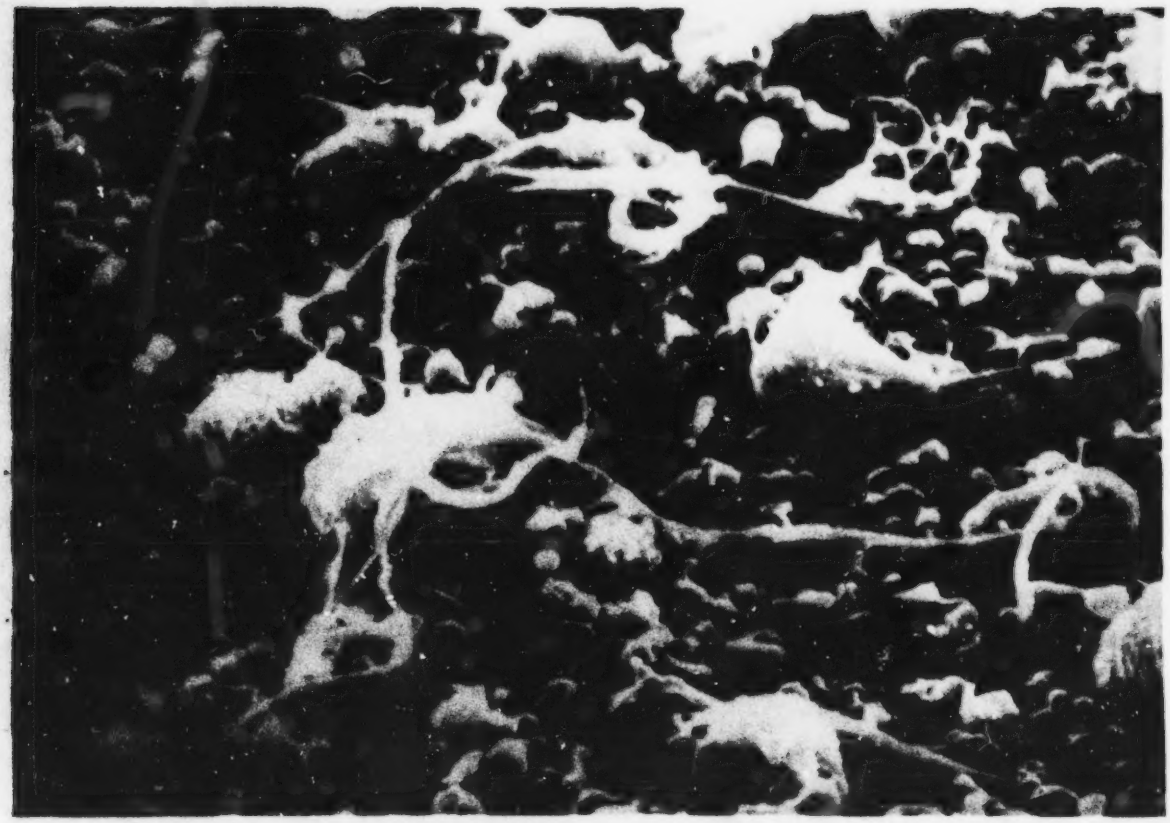
Figures 14 & 15 show examples of organisms from culture JC-3, prepared as for Figures 9 - 13.

The identification/classification of the various organisms in these cultures was not attempted at the present time. There was a diverse range of organisms present in these cultures but flagellated species appeared to predominate. Based on the magnifications used, none of the organisms in the photographs is greater than 10 microns in length or diameter (excluding the flagella). In most cases, dimensions of 2 - 4 microns are more typical. During examination in the optical microscope, it was estimated that some of the organisms in cultures JC-3 and 3A-2 were in the size range 20-40 microns. However, none of these larger forms were detected in any of the electron microscope studies. The apparent small size of the organisms in these cultures fitted well with their intended introduction into the fine particulate environment of a tailings pile.



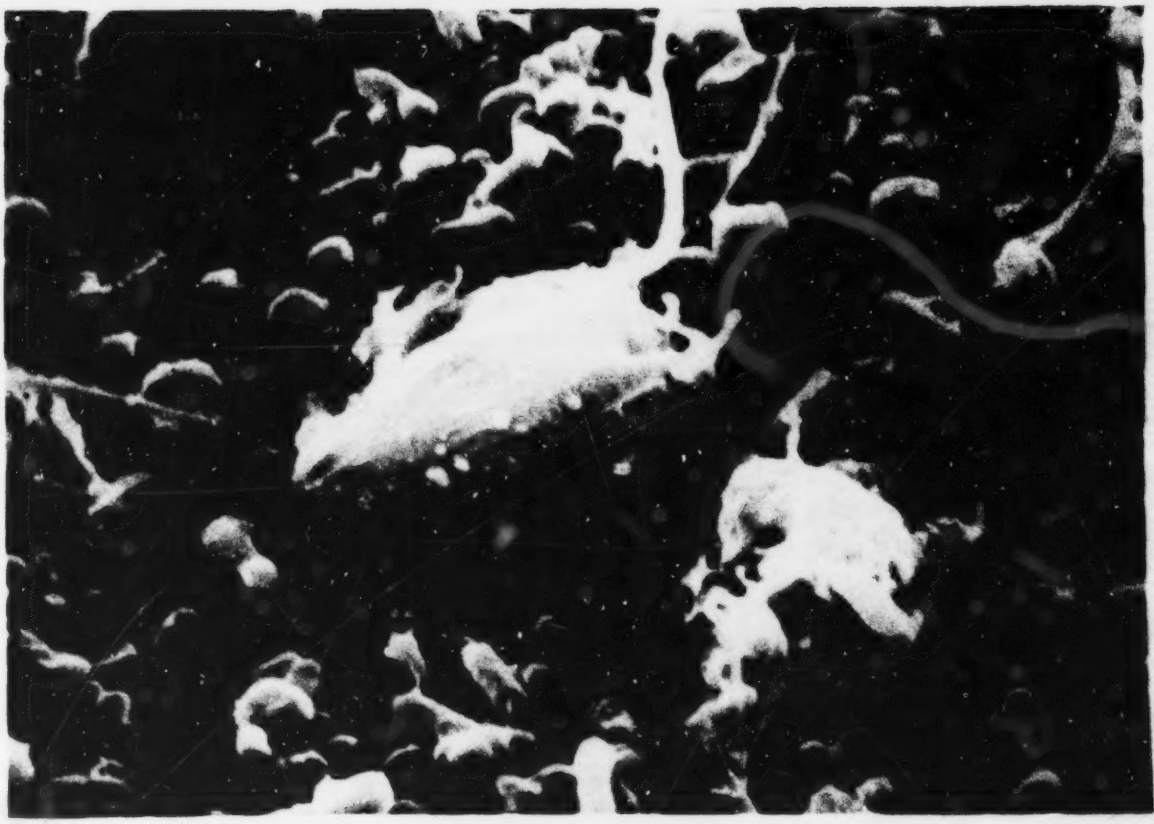
Scale - 3 µM

Figure 6: Protozoa present in culture 3A-2 with bacterial cells in background. Scanning electron microscopy



Scale - 3 uM

Figure 7: Protozoa from predator culture 3A-2 with bacterial cells in background, at higher magnification. Scanning electron microscopy.



Scale - 3 µM

Figure 8: Protozoon from predator culture 3A-2, with bacterial cells in background, highest magnification. Scanning electron microscopy.

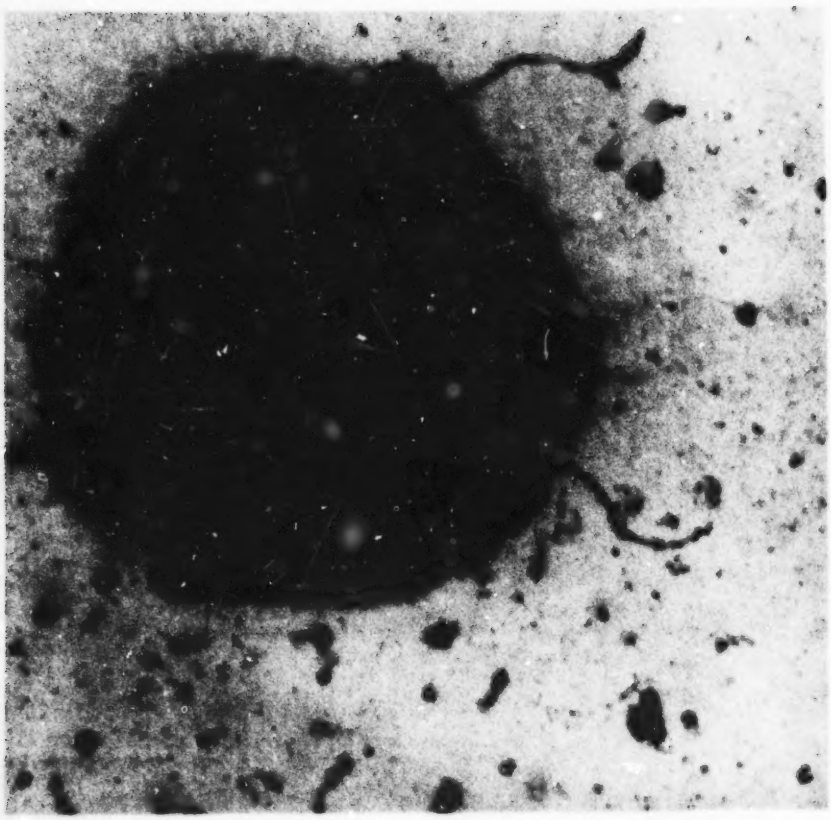


Figure 9: Protozoon from predator culture 3A-2. Magnification x 17,000. Transmission electron microscopy.

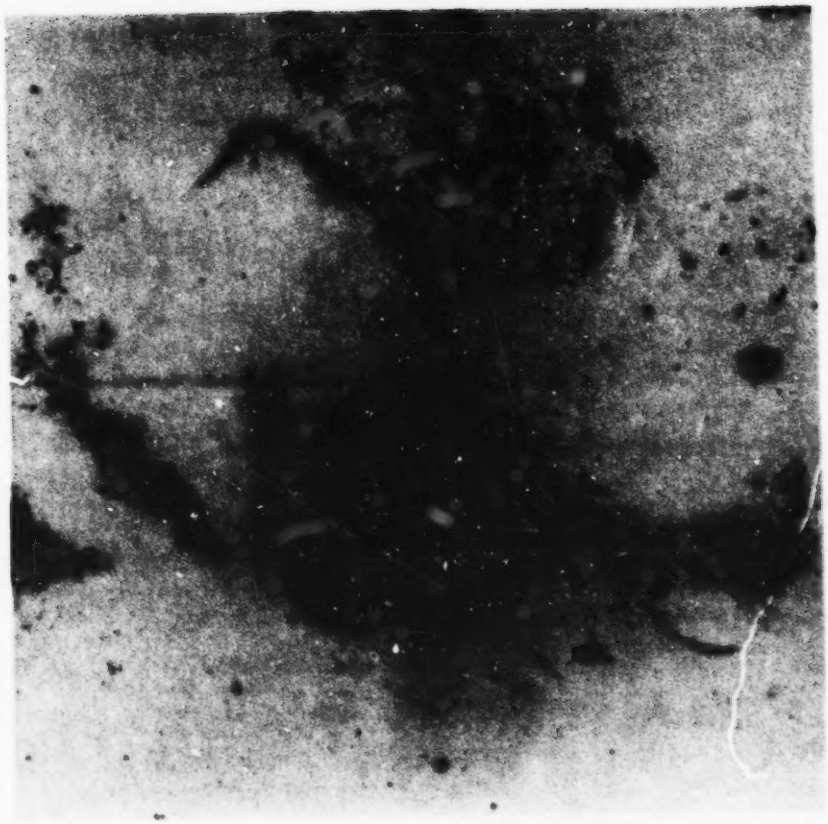


Figure 10: Protozoon from predator culture 3A-2. Magnification x 21,000. Transmission electron microscopy.

Figure 11: Protozoon from predator 3A-2. Magnification x 10,000. Transmission electron microscopy.

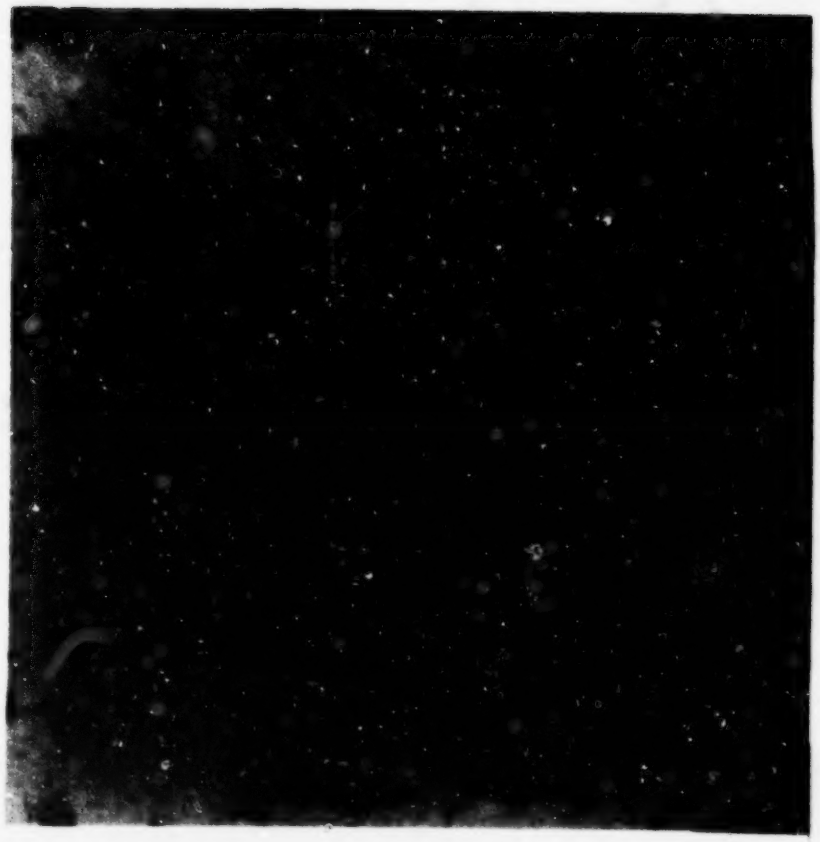


Figure 12: Protozoon from predator 3A-2. Magnification x 39,000. Transmission electron microscopy.

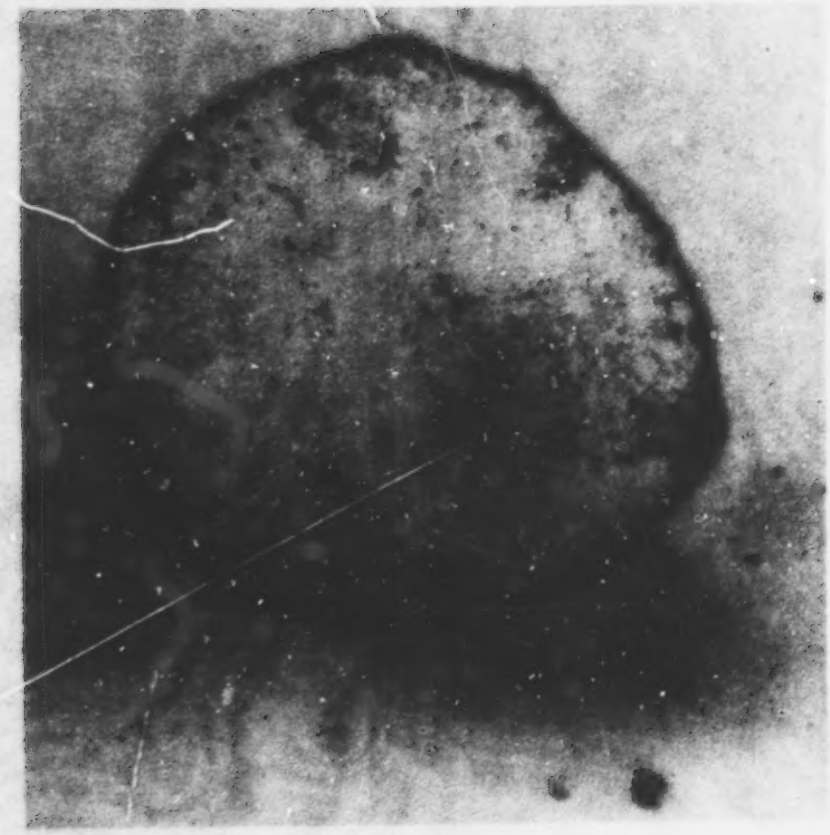


Figure 13: Protozoon from predator culture 3A-2. Magnification x 17,000. Transmission electron microscopy.

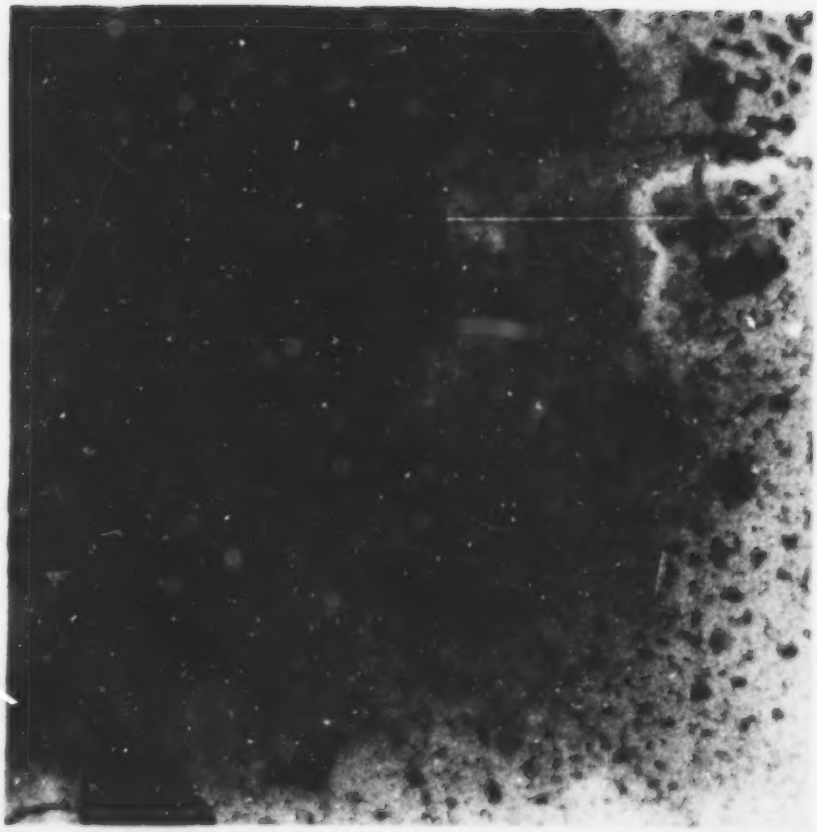


Figure 14: Protozoa from predator culture JC-3. Magnification x 17,000. Transmission electron microscopy.

The emphasis placed on alternative culture techniques was not solely due to the potential labour and cost savings from finding a replacement for T. ferrooxidans. The eventual goal of this feasibility study was to evaluate the ability of the predatory organisms to inhibit the growth of T. ferrooxidans in sulphide-containing tailings. To be effective, it was considered desirable to be able to eventually inoculate tailings samples with the greatest possible numbers of the predators. Growth on T. ferrooxidans was limited by the apparent minimal number of prey required. This would mean that inocula applied to the tailings would be introducing significant numbers of T. ferrooxidans cells. Although not acclimated to the tailings, the potential effect of these additional bacteria could not be ignored. One solution would be to be able to culture the organisms in "clean" media so that the predators could be concentrated prior to their application to tailings. Similarly, the effect of constituents in the bacteria-based culture media other than the T. ferrooxidans and the predator, on the acid-generating within the test tailings could be monitored more easily with the use of relatively clean culture media.

4.4 Lysimeter Tests on Pyrrhotite Tailings

Predator culture 3A-2 was selected for this phase of the study. Although culture JC-3 appeared more attractive for use at a higher pH value, the assumption was made that at many mine sites with acid-generating tailings, the pH of the acid rainwater is commonly close to a value of 4.1. Thus, a candidate organism must be able to survive at the pH range likely to be created by the leaching of tailings with rainwater. This requirement eliminated the JC-3 culture at the present time. The goal of the application of predators will be to control the T. ferrooxidans at the pH values (i.e., less than 4.0) where the bacterium will begin to show more active growth and acid-generating activity.

Sulphide-containing tailings from three sources were evaluated. Two of these samples, from Falconbridge and Kidd Creek Mines, respectively, were found to be of an extremely fine particle size distribution. The data (Table III) for the particle size distribution of a dried Falconbridge

TABLE III: PARTICLE SIZE DISTRIBUTION OF DRIED FALCONBRIDGE TAILINGS

FRACTION (μM)	WEIGHT (%)
+ 149	39.0
- 149 + 106	8.0
- 106 + 75	11.5
- 75 + 44	10.0
- 44	31.5

tailings sample show a high percentage of the particles to be less than 44 microns in diameter. Samples of the predators were mixed with the wet tailings and examined under the microscope. In most instances, the predators in the cultures were equivalent in size to many of the particles. Thus, the movement of the motile predators into the finer tailings was expected to be limited. Accordingly, to enable this preliminary assessment to be completed, an alternative pyrrhotite tailings sample was obtained from Inco Ltd. These tailings were much coarser than the previous samples and were a demonstrated source of acid. Strong cultures of T. ferrooxidans were obtained when the Inco tailings were inoculated into 9K medium. The "natural" pH of the discharge from the tailings as received was approximately 2.2-2.4.

Two series of tests were conducted on the pyrrhotite tailings. In the first instance, a single inoculation of predator culture 3A-2 was applied to the test lysimeter boxes. An estimated 2.5×10^6 protozoa were applied in 50 mL of solution to the test lysimeters which had previously been "neutralized" to pH greater than 4.0 with alkali and extensive washing with water. The pH of the seepage collected from the lysimeter boxes when compared with the control tests (no inoculum or heat inactivated inoculum) showed no signficant difference (Table IV). In all instances, the pH of the discharge decreased steadily to approximately 2.6-2.9 over a period of 17 days. It was assumed that the predator was not inhibiting the establishment of an acid-generating bacterial population and therefore, the test was stopped.

In the second test, the acidity within the tailings in each lysimeter box was again partially neutralized to pH greater than 4.0. Six (6) applications of predator culture 3A-2 were made to the test columns during the first 15 days of the total test period (31 days). The data (Table V) for the pH of seepage from each lysimeter box indicate that compared with the previous tests, the acid generation within the tailings is slightly less than in the previous experiment and has been partially inhibited. However, the unusual observation was that such inhibition was noted with all lysimeter tests (controls and tests). By Day 21, the pH values for seepages from all six lysimeters were in the range 2.8 - 3.1 and remained there for the remainder of the test.

TABLE IV: LYSIMETER EXPERIMENTS ON PYRRHOTITE TAILINGS - TEST 1 ①

Day	Lysimeter Discharge pH ②					
	1	2	3	4	5	6
1	3.4	4.0	6.3	3.9	3.4	3.2
3	3.4	3.6	5.5	3.8	3.2	3.0
4	3.4	3.7	5.7	3.4	3.1	3.1
6	3.2	3.6	5.0	3.4	3.0	3.0
7	3.3	3.5	3.5	3.3	3.0	3.1
10	3.0	3.4	3.6	3.4	3.0	3.1
11	3.0	3.3	3.6	3.2	2.9	3.1
14	3.0	3.2	3.3	2.9	2.7	3.0
17	2.8	3.0	2.9	2.7	2.6	2.8

① Lysimeters 4 and 5 inoculated with predator culture 3A-2 on Day 0.

Lysimeters 2 and 3 inoculated with inactivated predator culture 3A-2 on Day 0.

② Seepage volume - 0.14 L/lysimeter/day
Distilled water pH 4.1 was added as make-up to lysimeters 1, 2, and 5.
Tap water pH 4.1 was added as make-up to lysimeters 3, 4, and 6.

TABLE V: LYSIMETER EXPERIMENTS ON PYRRHOTITE TAILINGS - TEST 2

DAY	LYSIMETER DISCHARGE pH (1)						T. FERROOXIDANS NUMBERS IN DISCHARGE (2)						WATER ADDITION [L] (3)					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
0*																		
1							$>10^4$	10^2	$>10^4$	10^4	$>10^4$	$>10^4$						
3*																		
6*	2.8	5.1	3.2	3.3	3.1	3.1			$>10^4$		$>10^4$		0.30	0.30	0.30	0.30	0.30	0.30
7	2.8	5.0	3.3	3.4	3.1	3.2							0.14	0.14	0.14	0.14	0.14	0.14
8	2.8	4.3	3.3	3.3	3.1	3.1	$>10^4$	10^2	10^3	10^3	10^3	10^3	0.30	0.30	0.30	0.30	0.30	0.30
9	2.9	3.8	3.2	3.3	3.1	3.1							0.30	0.30	0.30	0.50	0.50	0.30
10*	2.9	3.6	3.1	3.3	3.0	3.1	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	0.15	0.15	0.15	0.15	0.15	0.15
13*	2.7	3.2	2.8	3.1	2.9	2.9	10^2	10^2	10^3	10^2	$>10^4$		0.40	0.40	0.40	0.40	0.40	0.40
14	2.6	3.2	2.9	3.0	2.5	3.0							0.10	0.10	0.10	0.10	0.10	0.10
15*	2.5	3.2	2.9	3.0	2.6	2.9	$<10^2$		$>10^4$		$>10^4$		0.30	0.30	0.30	0.30	0.30	0.30
16	2.6	3.2	2.9	2.9	2.6	3.0							0.30	0.30	0.30	0.30	0.30	0.30
17	2.6	3.2	2.9	3.0	2.6	2.6	10^4	10^4	10^3	$>10^4$	$>10^4$	$>10^4$	0.14	0.14	0.14	0.14	0.14	0.14
20	2.5	3.1	2.8	3.0	2.5	2.6							0.60	0.60	0.60	0.60	0.60	0.60
21	2.7	3.0	2.8	2.8	2.8	2.9							0.40	0.40	0.45	0.50	0.50	0.50
22	2.8	3.0	2.8	3.1	3.0	2.9							0.10	0.10	0.10	0.10	0.10	0.10
23	2.8	3.0	2.8	3.0	2.9	2.9							0.25	0.35	0.25	0.25	0.25	0.35
24	2.9	3.0	2.9	3.1	3.0	2.9							0.20	0.20	0.20	0.20	0.20	0.20
27	2.9	3.0	2.8	3.0	2.9	2.9							0.30	0.30	0.30	0.30	0.30	0.30
28	2.9	3.0	3.0	3.1	3.0	2.9							0.30	0.30	0.30	0.50	0.50	0.30
29	3.0	3.0	2.9	3.0	2.9	3.0							0.25	0.25	0.25	0.25	0.25	0.25
30	3.0	3.0	2.9	3.0	2.9	3.0							0.15	0.15	0.15	0.15	0.15	0.15
31	3.0	3.0	2.9	3.0	2.9	3.0												

(1) Seepage volume - 0.14 L/lysimeter/day
(2) Estimated from 5 tube 3 dilutions MPN test, 10 day incubation period.
(3) Lysimeters 1, 2, 5 and 6 received distilled water pH 4.1
Lysimeters 3 and 4 received tap water pH 4.1
* Lysimeters 4 and 5 inoculated with predator culture 3A-2
Lysimeters 2 and 3 inoculated with inactivated predator

Control lysimeters #2 and #3 (inoculated with heat inactivated predator) did not give a pH value less than 2.8 throughout the test. A similar pattern was noted with lysimeter #4, inoculated with predator culture and tap water pH 4.1 or make-up. However, during the course of the test, both uninoculated controls (lysimeters #1 and #6), as well as test lysimeter #5 (predator + distilled water pH 4.1 make-up) showed a pH value for the seepage as low as 2.5 - 2.6. However, these lower values were not maintained and over the course of the next few days, the pH values of these seepages gradually rose to a value of approximately 3.0. Such adjustment occurred at the time that active predators were not being introduced into the lysimeters #4 and #5..

During the course of this experiment it was estimated that in excess of 10^6 protozoa were added to each of the test lysimeters (#4 and #5) together with more than 10^9 T.ferrooxidans cells. Control lysimeters (#2 and #3) receiving heat-inactivated predator also received additional T. ferrooxidans cells but to a lesser extent due to some loss of viable T. ferrooxidans cells during the pH/heat inactivation of protozoa (approximately 10^6 cells/mL in a predator culture was reduced to approximately 10^4 cells/mL by the treatment).

Estimates of the numbers of T. ferrooxidans in the lysimeter discharges were calculated from the fifteen tube (3 dilutions/5 tubes) most probable number (MPN) tests in 9K medium based on an incubation period of 10 days. At longer incubation periods (up to 15 days), all dilutions were generally positive indicating bacterial numbers in excess of 10^4 cells/mL of seepage solution. After 10 days' incubation, there was still some difference between the cultures in the rate of development of the iron-oxidizing behaviour. Estimating the bacterial numbers at this time rather than waiting for an incubation period of fifteen days or longer, affords comparative data but will mean that the values for the estimated bacterial numbers as shown in Table V, will be low by several orders of magnitude.

The data over the first sixteen days of the test proved to be erratic with no consistent patterns being detected. The enumeration tests were therefore discontinued. These variations were not unexpected since the major fraction of the T. ferrooxidans within any one lysimeter might be expected to be associated with the particle surfaces. The MPN test indicated that the T. ferrooxidans introduced with predator culture did not appear to cause a significant change in the numbers of bacteria in the discharge when compared with the control lysimeters (no inocula). These control lysimeters represent the natural T. ferrooxidans population within the tailings. The estimated numbers of bacteria in the lysimeter discharges, (10^2 - 10^4), would not appear adequate to maintain the protozoa in active vegetative forms within the interstitial spaces of the tailings. However, the number of T. ferrooxidans associated with the particles was an unknown factor as was also the ability of the predators to prey on these attached organisms.

At Day 14 of the incubation period, samples were withdrawn from the liquid within the tailings samples. These were examined microscopically for the presence of protozoa. At the outset of the program no motile protozoa were detected in any of the samples from the lysimeter boxes. At Day 14, protozoa were detected in the samples from lysimeter box #5. The motile organisms were very active and resembled the organisms present in the original inoculum of 3A-2. No protozoa were detected in the samples from the other lysimeter boxes.

At Day 21 samples from liquid within the lysimeters were again examined. Lysimeters #4 and #5 contained active, motile protozoa, (estimated population 100 organisms/mL). The organisms in lysimeter #4 resembled the original 3A-2 culture but in lysimeter #5, the dominant protozoa were two large ciliated species. One of these (greater than 100 microns in length) resembled members of the Oxytrichidae family, whereas the other had an oval shape (approximately 30 microns long). No protozoa were detected in samples from lysimeters #1, #2, and #3.

A similar pattern was noted in samples taken on Day 27 with the active large ciliated protozoa being present in the test lysimeters but none detected in the controls. However, in the samples collected on Day 28, active protozoa were detected in samples from each of the lysimeter boxes. These protozoa were all large ciliated species similar to those described above. No flagellated organisms were detected. In the final samples collected on Day 30, active protozoa were detected in samples from lysimeters #1 - 5, but none from lysimeter #6. The large Oxytrichidae-type ciliate predominated with occasionally the smaller oval, ciliated species being detected. In #5, one organism similar to the form present in the original 3A-2, was observed.

Repetitive subsampling indicated that the number of organisms in each sample was low, i.e., less than 20/mL. The predominance of these larger organisms and their presence in the control lysimeters suggests that they were either present in the original tailings (without being detected) or alternatively, they have been introduced in the make up water. These larger species may be predators of the smaller protozoa inoculated into the test columns in culture 3A-2.

5. CONCLUSIONS

In this preliminary evaluation, a range of environmental samples have been screened for the presence of protozoan predators able to utilize T. ferrooxidans as prey. It has been demonstrated that protozoan predators can be isolated which will prey on T. ferrooxidans. Morever, these predators can be active at low (2.5 - 3.5) or higher (4.5 - 6.2) pH values and include smaller protozoan forms, e.g. flagellates, considered to be of more practical significance to the intended end use.

Culture 3A-2, selected for lysimeter tests on tailings, consisted of a mixed population of protozoa. These organisms were relatively small in size, i.e., less than 5 microns in diameter/length. The protozoan population consisted predominantly of flagellated organisms which were highly mobile. This small size was considered important to allow access by organisms to interstitial spaces between fine particles. Nevertheless, certain types of tailings of very small particle size may not be suitable. This factor requires further investigation.

Small-scale lysimeter tests to evaluate the ability of the protozoan culture 3A-2 to control acid generation in tailings suggest that some level of control was achieved with the use of multiple applications.

The detection of larger ciliated organisms in all lysimeter boxes poses some questions which cannot be answered by the current tests. These organisms may have been introduced in the original tailings or the make up water. They may also be predatory towards the smaller flagellated protozoa introduced into the test columns in the predator culture. This development represents one of the possible sequences that could be observed in the natural environment.

The data are still inadequate to develop a control technology using biological agents. There are many unanswered factors including whether or not the bacterial concentrations within tailings are high enough to support directly the vegetative growth of those predators. It may be necessary to support the growth of predators within tailings by the supplying of other sources of bacterial prey. Particular benefits would result from being able to add bacterial species for which the predator cultures show a lower level of preference than for T. ferrooxidans. Thus the alternate prey would only be utilized in situations where T. ferrooxidans numbers become limiting.

The use of smaller predatory organisms appears to match more closely the physicochemical environment of a tailings pile. These smaller organisms may be expected to require lower numbers of bacterial prey for individual survival and growth. A control strategy may depend on being able to introduce an adequate number of the predator into a tailings pile.

The search for more active "killer" organisms should be continued. However, the more active the predatory behaviour of a culture, the more difficult it is to maintain and culture the organisms in large quantity. It requires a large number of cells and if T. ferrooxidans is the sole prey, it will be a very expensive factor. Alternative culture techniques must be investigated.

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Similarly, for more conclusive tests, a method is required to separate and concentrate the protozoa from the bacterial prey in cultures. The introduction of large numbers of T. ferrooxidans with the predators is not desirable. However, the close similarity between the size of these predators and their bacterial prey makes the task of separating them a difficult one. Again, alternative culture techniques may offer a partial solution to this problem.

We conclude that biological control mechanisms deserve further investigation. There are many interfering and as yet, unknown factors which must be examined more extensively. This applies to the use of the protozoan predators. At the same time, other control methods proposed at different times in the literature, e.g., the use of Caulobacter spp., should not be ignored. They also require more extensive evaluation than has been carried out to-date.

6. RECOMMENDATIONS

It is recommended that future studies concentrate on:

1. Larger-scale lysimeter tests to simulate more closely, the flow patterns of the field situation.

2. The investigation of improved techniques to culture larger quantities of predator organisms to be used in the lysimeter tests.

3. A continued screening program to identify more effective predator species.

4. A preliminary laboratory investigation of the use of Caulobacter species as inhibitors.

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APPENDIX I

APPENDIX I

ALTERNATIVE CULTURE MEDIA INVESTIGATED

1. Medium for Colorless Flagellates (Hall, 1937)

Modified Medium G

Glycine (2.0 g), glucose (2.0 g), sodium acetate (2.0 g), dipotassium hydrogen phosphate (0.2 g), and magnesium sulphate (0.1 g) were dissolved in distilled water (1 L). The pH was adjusted to 5.3 with acetic acid and the solution filter sterilized.

2. Media for Ciliates (Hall, 1937)

Medium N

Magnesium sulphate (0.025 g), calcium chloride (0.001 g), potassium nitrate (0.5 g), ammonium phosphate (0.05 g), and ferric chloride (0.001 g) were dissolved in distilled water (1 L). The pH was 6.2 and the solution was sterilized by autoclaving at 121°C.

Medium P

Potassium dihydrogen phosphate (0.002 g), sodium chloride (0.02 g), magnesium sulphate (0.02 g), calcium nitrate (0.29 g) and ferrous sulphate (trace) were dissolved in distilled water (1 L). The pH was 5.9 and the solution was sterilized by autoclaving at 121°C.

3. Hay Infusate

Approximately 8 dozen pieces (1" long) of fresh hay were added to distilled water (1.5 L) and the mixture was boiled gently for 45 min. Magnesium sulphate heptahydrate (0.1024 g), potassium nitrate (0.51 g),

calcium chloride dehydrate (0.0026 g) and ammonium hydrogen phosphate (0.05 g) were then dissolved in the mixture. The infusate was cooled, diluted to 2 L with distilled water and filter sterilized (0.22 um filter).

4. Rice/Yeast Medium (Jackson and Berger, 1984)

Distilled water (100 mL) was sterilized. Five raw rice grains and 5 spheres of Fleischmann's dried baker's yeast were added to the sterile water.

APPENDIX II

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AN ASSESSMENT OF THE USE OF MICROBIAL ORGANISMS TO CONTROL NITRATE LEVELS IN PROCESS EFFLUENTS AND TAILINGS

FINAL REPORT
NO. 41-10246

Work on this project was conducted under the auspices of the Canada Centre for Mineral and Energy Technology, Energy, Mines and Resources, Canada.

DSS FILE NO: 26SQ.23440-3-9137
DSS CONTRACT SERIAL NUMBER: OSQ83-00197
CANMET SCIENTIFIC AUTHORITY: MR. H.W. PARSONS

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April 1st, 1985

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SUMMARY

Biological denitrification processes have been reviewed relative to their potential application to the reduction of nitrate concentration in mining effluents.

The biological process designs include suspended growth systems, rotating biological contactors, packed bed reactors and fluidized-bed reactors. The application of the different types of denitrification systems to the treatment of industrial wastes, similar in composition to those in a mining environment have been assessed. These techniques were also compared with other nitrate-removal techniques including ion-exchange, reverse osmosis and chemical treatments.

It is concluded that biological denitrification is an attractive and generally more economical process to reduce the nitrate concentrations in discharge waters to the low concentrations required to meet environmental standards. Of the different types of reactor design, fluidized-bed reactors or rotating biological contactors appear most suited to the requirements of the mining industry.

The most predominant sources of nitrate ions in the mineral processing industry include wastewaters from underground mining and process waters from ion exchange (e.g., uranium) separations. Surface water quality criteria for nitrate and nitrite have been reviewed along with the current recommendations for mine discharges. Data on the present nitrate concentrations in discharges from different mines are very limited. From the data available, no serious health related problems could be identified. However, environmental concerns, i.e., eutrophication, toxicity to fish, etc. may necessitate the reduction of nitrate concentrations in discharges. Such problems will be site specific and dependent on factors such as the dilution rate in the receiving body of water.

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Key recommendations for future study include an expanded review of the nitrate concentrations in minewaters, an assessment of the occurrence of ecological problems related to the presence of nitrate, and a study to evaluate the efficiency of biological denitrification in the treatment of minewaters.

1. INTRODUCTION

The Ontario Research Foundation was awarded a contract in response to an unsolicited proposal by the Department of Supply and Services, to review the potential of biological denitrification as applied to the mining industry. This represents the extension of the contract DSS File No. 23 SQ.23440-3-9137 to study the Biological Control of *Thiobacillus ferrooxidans* by Predatory Organisms. The present report summarizes the biological denitrification processes and their potential application to the mining industry.

A literature review has been carried out on the biological denitrification process which involves the removal of nitrate ions from effluents and wastewaters by the action of micro-organisms. The eventual end product of the reaction is gaseous nitrogen or nitrogen oxides. Most of the published data describe combined nitrification/denitrification systems whereby ammonia is converted initially to nitrate ion (*i.e.*, nitrification) for subsequent denitrification. Similarly, the greater portion of the information in the literature refers to domestic wastewaters. In this report, we have attempted to translate the available information on denitrification processes so that their potential for application in the mining industry can be assessed. Since, ammonia is a common constituent of mining and mineral processing effluents and due to the difficulties in separating nitrification from denitrification stages in certain processes, some information has been included on the technology of nitrification. However, an in-depth review of the nitrification process as it might apply to the mining industry is beyond the scope of this report.

The objectives of this study are:

i) to assess the sources and level of occurrence of nitrate-containing wastewaters within the mining and mineral processing industries
ii) to examine the technical and economic feasibility of using biological denitrification processes to remove nitrates from such wastes.

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2. HEALTH AND ENVIRONMENTAL FACTORS

A wide variety of nitrogen containing chemicals are used in mineral processing operations including organic amines, ammonium salts, ammonium hydroxide, nitric acid and nitrate salts. Ammonium nitrate/fuel oil mixtures (AN/FO) are also widely used as explosives. This will result in the presence of nitrates, nitrites, ammonia and other nitrogenous compounds in the plant wastewaters. The nitrogen compounds of concern for this study are nitrate and nitrite. Water quality criteria for these compounds fall into two categories; those concerned with human and animal health and those concerned with the quality of the environment and aquatic life.

Drinking water standards for nitrate and nitrite have been established. The sole known clinical manifestation of nitrate ions present in drinking water is methemoglobinemia in infants. The sensitivity of children particularly below the age of six months to high nitrate concentrations in their food or water is attributed to "nitrate reduction" occurring in the intestines of an infant. The nitrite produced as a by-product of this reaction converts haemoglobin in the blood to methemoglobin. This latter compound is unable to carry oxygen in the bloodstream with the result that the child will suffer from cyanosis or suffocation. Although, a strong correlation appears to have been established between the presence of "high" concentrations of nitrates in drinking water and the occurrence of methemoglobinemia, there is still a lack of consensus of opinion as to what constitutes a "safe" concentration of nitrate in water for human consumption. The World Health Organization has set a maximum permissable limit of 45 mg/L of nitrate (NO_3) or 10 mg/L as nitrogen (N) (World Health Organization, 1978). This standard was also adopted by Canada (Health and Welfare Canada, 1978) with the 10mg/L as nitrogen applying to the total nitrate and nitrite concentrations.

Nitrate has a relatively low toxicity towards fish and aquatic species which can in general tolerate concentrations in excess of 1000 mg/L NO_3-N. However, control is necessary to prevent eutrophication, excessive algal growth and oxygen depletion in receiving waters. It has been suggested

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that to prevent eutrophication the total inorganic nitrogen level (NO_3-N + NO_2-N + NH_3-N) should not exceed 0.3 mg/L in lakes and 1.0 mg/L in flowing rivers (U.S. EPA, 1973). However, the development of the eutrophication in any one particular situation will also depend on the availability of other nutrients such as phosphorus and other forms of nitrogen.

The maximum acceptable limit for nitrite ion should also be considered. In Canada, the standard has been set at 1.0 mg/L NO_2-N.(Health and Welfare Canada, 1978) and reflects the higher toxicity of the nitrite ion towards humans. This enhanced toxicity is also observed with aquatic species. Fish are very sensitive to nitrite ion and the toxic action resembles that in human infants. To protect fish populations, nitrite concentrations as low as 0.05 mg/L would appear to be desirable (U.S. EPA, 1976).

Nitrite ion may also originate from the microbial nitrification process in the natural environment by which ammonium ions are converted to nitrite ions. This reaction will usually continue with the eventual oxidation of the nitrite to nitrate species. The impact of this process is to reduce the dissolved oxygen levels in the water. If the process is inhibited by factors such as oxygen deficiency or low temperature, an accumulation of toxic nitrite ions in the water may occur.

The discharge of nitrogen and more particularly nitrates in mining and mineral processing effluents is not regulated at the present time, at least not at the Federal level. In Ontario, while objectives exist for nitrate and nitrite in drinking water, there are no comparable standards for the mining industry.

In British Columbia, final effluent objectives for the mining industry are 10-25 mg/L for (NO_3 + NO_2)-N. Current receiving water objectives are 10 mg/l NO_3-N and for nitrite the goal is an average of 20 mg/L NO_2-N with a maximum level of 60mg/L NO_2-N (British Columbia MOE, 1979).

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The ongoing debate on the detrimental effects arising from the release of low concentrations of nitrate ions into the environment will influence significantly the social and economic justification for a company to introduce control measures. The cost/benefit factor must be carefully assessed.

3. SOURCES AND LEVELS OF NITRATES IN MINING EFFLUENTS

Nitrates and nitrogenous compounds in general will originate in mining and mineral processing wastewaters from the use of nitrate based explosives as well as the nitrate chemicals used in mining and/or processing operations, e.g., elution of ion exchange resins. AN/FO (dry ammonium nitrate/fuel oil mixtures) and slurry/water gel explosives containing ammonium nitrate, calcium nitrate or sodium nitrate in an aqueous suspension comprise a large proportion of the explosives used in hard rock mining. This subject was reviewed in some depth by Pommen (1983) and only a few specific aspects will be highlighted in this section.

Pommen identifies that most of the concern regarding undesirable nitrate concentrations in minewaters originated with open pit mining procedures. The risk of water uptake by the AN/FO type of explosive is high and it is not suitable for use in wet boreholes. If the moisture content in the AN/FO exceeds 5% by weight, the explosive will apparently fail to detonate. When this happens, the unexploded mixture is dissolved in water and flushed out of the bore hole.

The slurry/gel mixtures are designed to be used in wet boreholes. An aqueous suspension containing the ammonium nitrate and fuel in the presence of a guar gum is added to a bore hole. This is followed by the addition of a crosslinking agent to cause the gel to set. The gel is said to exhibit a high resistance to water thus leaching of nitrate from the explosive prior to detonation is not considered a problem nor does the mixture fail to explode due to excess moisture.

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The ammonium nitrate concentration in AN/FO mixtures may be as high as 94% by weight. Ammonium nitrate is very soluble in water i.e. 1183 g/L cold water at $0^{o}C$ (Anon, 1983). Therefore, ammonium and nitrate ions will be introduced into minewater as a result of spills of explosives and misfires (direct dissolution of compounds), leaching of chemicals while the explosive is in a bore hole, residues in crevices in the bore holes or slurry deposited in the upper zones of the walls of the bore holes. In the last named instances, the chemicals will not be detonated in the explosion. Pommen indicates that in open mining situations as much as 6% of the total nitrogen in the explosives is discharged in effluents and that as much as 95% of the nitrogen in the effluents from an open pit coal mine originates from the explosives used.

Nitrogen, ammonia, nitrous oxide, nitric oxide and nitrogen dioxides may be present in the detonation gases. While most of these gases will be lost to the atmosphere (particularly in the case of the open pit processes), some absorption of gases into the minewater is expected to occur. This may be more significant when these explosives are used underground.

In the case of mineral processing effluents, the concentrations of target compounds will depend on their degree of use in the milling processes. In the uranium industry, for example, ammonium nitrate is used for the elution of ion exchange resins and ammonium sulphate is used for the organic stripping in solvent extraction. Nitrate may also be used in lead refining (Patton, 1984).

In many milling processes, effluent volumes can be reduced by the recycling and re-use of water. In other situations, nitrate usage can be eliminated or reduced by switching to alternative processes or reagents. The uranium industry offers a good example of this approach. Thus, sulphuric acid is used to strip the uranium from the tertiary amines at the solvent extraction stage in one instance (Eldor Resources), and is also used to elute resins.

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Alternative reagents and processes, which may be considered for different unit operations, must obviously be compatible with one another and the overall process. Alternatives may be more expensive to use and product quality may also be affected. In these situations, if nitrate concentration in the wastewater is a problem, consideration may be given to treating a particular bleed or stream, or even the plant or mine effluent by biological denitrification. As will be discussed later, applying the process to the smallest volume of solution may offer economic advantages due to the lower requirement for nutrients.

Biological processes for the removal of nitrogen compounds from wastewater have been developed largely for the treatment of domestic wastewaters. Usually, these processes are used in tandem to initially oxidize the ammonium ions and organic nitrogen compounds to nitrate and nitrite ions (nitrification). In the subsequent denitrification stage, the nitrate and nitrite are converted to gaseous nitrogen compounds including molecular nitrogen, nitrogen oxides etc. However, the processes may be applied separately to specifically remove ammonia/organic nitrogen or nitrate/nitrite, or in combination for the complete removal of nitrogen.

Denitrification processes are well documented in their application to the treatment of domestic wastewaters. Different processes have also been tested for the biological denitrification of industrial effluents but, with the exception of the new plant at National Lead, they have not found application in the mining or mineral processing industry.

It may be questioned if a need does in fact exist for denitrification processes in the mining industry. In many mines or mineral processing operations, the only source of nitrate to the environment will be from reagents such as the explosives. How significant is the contribution from this source is not well documented. There does not appear to be an adequate amount of published data on the levels of nitrate, nitrite and ammonium ions in mining and mineral industry wastewaters. Some data are presented in Table I. These data refer to levels existing before 1982 and in the case of nitrate values, the data are very limited.

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TABLE 1

NITROGEN LEVELS IN SOME CANADIAN MINE AND MILL EFFLUENTS

MINE	DESCRIPTION	CONCENTRATION (mg/L) NH_3-N	$(NO_2 + NO_3)$-N
Afton Mines		No elevated nitrogen levels	
Brenda Mines	Waste rock drainage	Unknown	Unknown
Coleman Mines	Mine water	8.2 - 9.6	Unknown
Denison Mines	Tailings effluent	31 - 70	Unknown
	Mine water	35 - 114	Unknown
Endako	Tailings effluent	2.3 - 3.8	Unknown
Equity Silver	Tailings effluent	1.3 - 1.7	Unknown
Fording Coal	Clode pond decant	0.36	8.6
	#8 Spoil drainage	0.49	76.2
	South Tailings Pond supernatant	6.8	8.8
Garson	Mine water	3.1 - 5.7	Unknown
Hudson Bay Mining and Smelting			
- Flin Flon	Tailings effluent	0.04 - 1.44	Unknown
- Snow Lake	Tailings effluent	0.02 - 1.8	Unknown
INCO			
- Copper Cliff	Tailings effluent	28.5 - 54	Unknown
- Levacle	Tailings effluent	2.6 - 6.5	Unknown
- Thompson	Tailings effluent	0.02 - 0.15	Unknown
Kidd Creek	Tailings effluent	0.1 - 1.5	Unknown
Lornex Mine	Tailings pond supernatant	Unknown	4.55(TKN=5)
	Pit water	Unknown	4.53
Mattabi	Tailings effluent	0.3 - 0.7	Unknown
Newmount		No recent elevated N levels	
NORANDA			
- Bell	Tailings effluent	0.06 - 0.94	Unknown
- Boss Mountain	Tailings effluent	0.2 - 2.2	1.6 - 2.0
Panel - P14	Tailings effluent	1.4 - 1.9	Unknown
	Mine water	29 - 135	Unknown
Placer	Tailings seepage	0.6 - 0.88	1.05
Quirke - Q3	Tailings effluent	30 - 108	Unknown
	Mine water	13 - 72	Unknown
Sherritt Gordon			
- Fox Lake	Tailings effluent	0.02 - 2.1	Unknown
- Ruttan	Tailings effluent	0.02 - 1.6	Unknown
Shamrock DS4	Tailings effluent	1 - 6	Unknown
Tantalum Mining	Tailings effluent	0.02 - 1.2	Unknown
Westmin Resources			
Buttle Lake	Tailings effluent	0.6 - 6.6	0.1 - 0.95

From Pommen (1983) and Blakeman (1984)

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4. THE MICROBIOLOGY OF DENITRIFICATION - A GENERAL OVERVIEW

4.1 Introduction

An extensive volume of literature has been published pertaining to biological denitrification and the biochemical/physiological mechanisms by which it takes place. Much of the microbiological literature refers to observations made in soil/sediment environments and therefore may not be directly applicable to wastewater systems. It is not intended to review herein, the literature pertaining to the biochemical pathways and theories. However, an attempt will be made to identify and highlight those factors which may relate to the effectiveness of a biological denitrification process if applied to the treatment of mining effluents. In some sections, the references quoted will direct the reader to selected review articles which document in greater detail information and variables collected from earlier literature (Christensen and Harremoes, 1977; Knowles, 1982; Payne, 1981, 1983).

4.2 Denitrification vs. Nitrification

Denitrification is identified for the purpose of this study as the dissimilative, biologically-catalyzed reduction of nitrate and nitrite ions to form gaseous nitrogen and/or nitrogen oxides. Dissimilative means that the bacterial cells do not convert the nitrate into cellular constituents. It is a facultative process inherent in certain micro-organisms whereby these organisms, which prefer to grow in the presence of oxygen (i.e., aerobically), are able to continue to respire in an oxygen-deficient (anoxic) environment.

In traditional waste treatment systems concerned with the reduction of the nitrogen content of waste streams, the denitrification stage is often preceded by a nitrification reaction in which ammonia and the reduced inorganic nitrogen compounds are first oxidized to nitrate ion. Although our principle concern in this study is the denitrification process. We should, however, consider briefly the difference between the two processes.

Nitrification is a process whereby a unique group of bacteria derive their energy for growth solely from the oxidation of ammonium or nitrite ions with the resultant formation of nitrite or nitrate ions respectively. These bacteria cannot utilize organic carbon and depend on the assimilation of carbon dioxide, carbonates and bicarbonates for their sources of the carbon required for synthesis of cellular components. They also do not utilize any other inorganic compounds as energy substrates, i.e., they are strict autotrophs depending for energy on the oxidation of inorganic nitrogen compounds. The most common species identified in nitrifying cultures are usually from the Nitrosomonas or Nitrobacter genera (Sharma and Ahlert, 1977).

Denitrification, on the other hand, is affected predominantly by heterotrophic organisms which derive their energy for growth from the metabolism of organic carbon compounds. These oxidative metabolic cycles generate reduced co-factors, which, in order to sustain the reactions, must be regenerated in the oxidized form. In aerobic environments, molecular oxygen is the terminal electron acceptor or oxidizer. When a select group of bacteria, the denitrifiers, find themselves in an oxygen-deficient environment they possess the ability to utilize oxidized inorganic nitrogen species (nitrate, nitrite, nitrous oxide) as alternate terminal electron acceptors. Gaseous nitrogen or nitrogen oxides are the by-products. This is a dissimilative reaction in that the nitrogen compounds are not taken up by the cells for the purpose of forming cellular constituents.

The denitrification process is considered to involve a series of enzyme-catalyzed reactions. Initially, nitrate ion is reduced to nitrite which is subsequently reduced via nitric oxide and nitrous oxide as intermediates to molecular nitrogen. The generally accepted pathway is therefore (Payne, 1973):

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$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The enzymes able to catalyze each of the above reactions have been extensively studied and characterized and the reader is referred to Thauer et al.(1977), Payne (1981) and Knowles (1982) for more detailed information.

4.3 Denitrifying Bacteria

Bacterial genera, which have been identified to include denitrifying species have been documented (Gamble et al., 1977; Ottow, 1980; Payne, 1973, Payne 1981, Knowles, 1982, Payne, 1983) and representatives are listed in Table II. They represent a very diverse group of organisms.

Denitrifying bacteria are predominantly heterotrophic although autotrophic denitrifying species such as Thiobacillus denitrificans (Baalsrud and Baalsrud, 1954) and photo-organotrophic species such as, Rhodopseudomonas spp. (Satoh, 1977) have also been identified. An additional noteworthy feature is the ability of a few Azospirillum spp. (Neyra et al., 1977), Rhizobium spp. (Zablowicz and Focht, 1979) and Rhodopseudomonas spp. (Sawada and Satoh, 1983) to fix elemental nitrogen. Similarly some Alcaligenes spp. (Castignetti and Hollocher, 1982) can both nitrify and denitrify heterotrophically depending on the environmental conditions.

Denitrifiers also vary with regard to substrate specificity. Some reduce nitrate and nitrite to molecular nitrogen. Others, however, may lack one or other of these metabolic pathways and reduce specifically only nitrate or nitrite. It is not surprising, therefore, that denitrifying systems will generally consist of a synergistic group of major and minor denitrifying species able collectively to achieve a high conversion of nitrate to nitrogen and other gaseous nitrogen oxides. However, the species commonly found in treatment systems are usually members of the Pseudomonas or Alcaligenes genera (Gamble et al., 1977; Payne, 1983).

TABLE II: MICROBIAL GENERA WITH DENITRIFYING SPECIES

GENUS

Achromobacter
Acinetobacter
Agrobacterium
Alcaligenes
Aquaspirillum
Azospirillum
Arthrobacter
Bacillus
Branhamella
Chromobacterium
Corynebacterium
Cytophaga
Flavobacterium
Gluconobacter
Halobacterium
Hyphomicrobium
Janthinobacterium
Kingella
Lysobacter
Moraxella
Neisseria
Paracoccus
Propionobacterium
Pseudomonas
Rhizobium
Rhodopseudomonas
Simonsiella
Thermotrix
Thiobacillus
Thiomicrospora
Vibrio
Xanthomonas

From Payne, 1973; Payne, 1983; and Knowles, 1982.

Denitrifying bacteria occur widespread in the natural environment (soils, water and sediments). It was estimated that about 10% of all soil bacterial species isolated are denitrifiers (Payne, 1983). As noted above, most of the studies to-date relating to waste treatment systems have centered on the use of mixed cultures of denitrifying organisms isolated by a process of natural selection. The carbon source used in the acclimation or enrichment process can influence which bacterial species or genera will predominate in an

isolate (Blaszyck et al.,1980; Sperl and Hoare, 1971). Thus, Hyphomicrobium spp. were found to predominate when methanol was used in combination with a high nitrate concentration in an enrichment medium (Sperl and Hoare, 1971; Nurse, 1980; Timmermans and Van Haute, 1983). The dominant species in natural isolates are also influenced by temperature and at lower temperatures, e.g., 2 - 5°C, psychrophilic species will prevail (Halmo and Eimhjellen, 1981).

4.4 Cultivation

4.4.1 Nutrition

As noted previously, denitrification is related directly to the carbon metabolism of the bacteria. An adequate source of soluble organic carbon is therefore an essential requisite for efficient denitrification systems. As an approximate guideline, it is generally accepted that the mole ratio of carbon to nitrate-nitrogen should be in the range 1.3 - 1.5. Since many industrial or mining waste liquors of high nitrate concentration do not contain sufficient soluble carbon, a low nitrogen, high carbon supplement is needed. The most often used carbon additive is methanol (Nurse, 1980; Timmermans and Van Haute, 1983), at typically 3.0 Kg methanol per Kg nitrate removed (Monteith et al., 1980) but other defined carbon nutrients such as acetate, ethyl alcohol, sucrose, and acetone have been proposed (McCarty et al., 1969).

High nitrate content, high volume wastes will require significant amounts of carbon supplement. The cost of this nutrient is obviously a major economic factor in the operation of a waste treatment denitrifying system. It will be particularly relevant for the high volume waste solutions generated in a mineral processing operation. Of the pure chemical supplements, methanol appears to be the least expensive. Nevertheless, considerable attention has been directed at finding more economical carbon supplements than methanol. Industrial waste liquors of high carbon content have been evaluated (Table III). Alternative carbon supplements, which have been investigated, include yeast, corn stillage, whey, spent sulphite liquor (Skrinde and Bhagat, 1982; Bridle, 1982) as well as brewery and distillery wastes, fusel oils and product streams from other industrial processes (Monteith et al., 1980) These

highchemical oxygen demand (COD) industrial wastes were all found to support active denitrification reactions. The rates of denitrification tend to be as good as or better than with methanol and the overall efficiencies of denitrification with these carbon supplements are still generally in the range of 80 - 90%.

TABLE III: ALTERNATE CARBON SOURCES*

Acetic Acid
Acetone
Brewery Waste
Chemical Process Wastes
Citrate
Corn Starch/Wastes
Ethanol Distillery Wastes (Fusel Oil)
Glucose
Lactate
Methane
Milk Solids
Molasses
Protein Compounds
Whey Sulphite Liquor
Winery Residues

* From Monteith et al., 1980; McCarty et al., 1969; Bridle, 1982.

The need for carbon (present in excess) can create undesirable results. During the denitrification reaction (80 - 90% effective), the carbon conversion/destruction is generally in the range of 30 - 50% although in one instance, with whey, the carbon conversion was as high as 79% (Skrinde and Bhagat, 1982). Generally, however, to destroy large amounts of nitrate it will be necessary to have present an excess concentration of carbon. Thus, the final effluents, while depleted as to nitrate, will contain substantial amounts of residual carbon. To meet environmental standards, it is usually necessary to carry out a secondary treatment of such effluents. An important

observation is that denitrification is sensitive to feedback inhibition from high substrate concentrations. Francis and Hancher (1981) concluded that NO_3 concentrations greater than 1.7 Kg/m^3 of NO_3-N (as NH_4NO_3) were inhibitory.

Denitrifying cultures acting on industrial and mining waste solutions may commonly require phosphorus supplements but these will not be as significant as the carbon requirements. Some organisms are also said to require certain trace elements such as copper and molybdenum. However, in mixed cultures selected by natural selection, the identification of such requirements will be a complex task. The addition of trace elements in the selection process may cause enrichment of some specific organisms having a requirement for that element, but whether there would be any gain in the rate and efficiency of the denitrification reaction over that of a culture not dependent on the presence of the element is difficult to predict.

The exception to the above requirements is the autotrophic denitrifying species Thiobacillus denitrificans. Although this organism does not require a carbon source for energy generation, an alternative energy source must be supplied for this organism in the form of a reduced sulphur compound. Typical substrates have included thio salts (Steinmuller, 1981; Yamauchi et al., 1981) and elemental sulphur (Neytzell-De Wilde, 1979; Steinmuller, 1981; Yamauchi et al., 1981).

4.4.2 Physicochemical Factors

Provided adequate amounts of carbon have been supplied to a high nitrate feedstock, the rate of denitrification is considered to be independent (zero order) relative to nutrient concentrations. At lower concentrations of substrate, it was contended that the rate of denitrification becomes first order relative to the nitrate. However, because of the close inter-relationship between the carbon oxidation and the nitrate reduction, it

is considered that the variations between zero order and first order kinetics are due to either carbon or nitrogen limitation (Focht and Chang, 1975). Therefore, provided that neither carbon or nitrogen are limiting, it is generally accepted that the kinetics of denitrification are zero order with respect to nitrate. The kinetics, however, very dependent on pH, temperature and oxygen tension.

4.4.2.1 pH

The optimum pH for a denitrifying culture is generally in the range 7 - 8. The precise operating pH will influence the composition of the gaseous end products. Thus, at pH values less than 7.3, nitrous oxide concentrations will increase relative to dimolecular nitrogen which is the main product at pH values greater than 7.3.

4.4.2.2 Temperature

Temperature is an important variable (Dawson and Murphy, 1972; Sutton et.al., 1975 ; Halmo and Eimhjellen, 1981). It is generally found that denitrification will occur over a wide temperature range (0 - 50^{o}C) with an optimum rate being observed at approximately 40^{o}C (Christensen and Harremoes, 1977). The rate of denitrification reactions is dependent on temperature. Christensen and Harremoes differentiate between short and long term temperature effects. Whereas the former has a direct impact on denitrification rates the latter condition introduces variables such as the adaptation of cultures.

The study by Halmo and Eimhjellen (1981) supported these observations. Concerned with the rate of denitrification at the low temperatures common in many temperate climates, they isolated two denitrifying cultures for a comparative study. The cultures, isolated by enrichment culture at 5^{o}C and 20^{o}C respectively, were identified as the low and high temperature sludges. In the former sludge, psychrophilic organisms (i.e., temperature optima for growth 15^{o}C) were predominant whereas mesophilic species (temperature optima generally 25^{o}C) were found in the high temperature sludge. Comparative tests of the denitrification rates at 0 - 17^{o}C (methanol as supplement) by the respective cultures indicated that the

low temperature culture gave 1.5 - 4 times higher rates of denitrification than the high temperature culutre. Above 17°C, the mesophilic culture was the more active. The significance of this observation is that depending on the ambient temperatures, it may be beneficial to isolate and maintain a culture at its routine growing temperature. Cultures isolated at higher temperatures in the laboratory may not be representative of the composition of the natural culture at the routine operating temperature in the plant. In the temperature range 0 - 17°C, the psychrophilic culture was also less affected by intermittent temperature fluctuations.

These authors also noted that with methanol as supplement, a mixed culture of denitrifying organisms was required for optimal rates of denitrification, i.e., a synergistic effect was present. Similarly, the settling characteristics of the low temperature sludge were better than for the high temperature sludge. Nitrification processes are inefficient (slow rates) at low temperatures. Thus any overall nitrogen removal process dependent on nitrification at the front end, will not function well at lower temperatures.

4.4.2.3 Oxygen Tension

Some observers regard oxygen as a toxicant to denitrifying cultures. In the sense that denitrification requires anoxic conditions, this is a reasonably accurate assessment. However, it is proposed that the toxicant label should be retained for combined oxygen forms in the cell rather than for the dissolved oxygen or oxygen tension restriction which will be regarded as a cultural requirement.

Denitrification requires anoxic conditions and the oxygen tension that can be tolerated by growing cultures is influenced by the culture environment. Thus the oxygen concentration for suspended cultures should be less than 0.5 mg/L (Christensen and Harremoes, 1977) or less than 0.4 mmol/L (Payne, 1983) Attached growth cultures, on the other hand, appear to tolerate higher dissolved oxygen concentrations (as high as 2 mg/L). It is presumed in the case of attached growth that the oxygen tension in the micro-environment within the biofilm is maintained below control levels by

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diffusion limitations (Christensen and Harremoes, 1977). It is important, therefore, to differentiate between measurements related to the macro- and micro-environments respectively. Certainly, if macro-concentrations are within tolerances, it is reasonable to presume that provided a combined oxygen source is not present, the conditions within the micro-environment will support denitrification. Intermittent or controlled access to oxygen is cited to have beneficial effects upon denitrification (Christensen and Harremoes, 1977).

The same limitations referred to for the dissolved oxygen measurements apply to the use of redox potential measurements to monitor denitrification reactions. If the redox potential is below - 200 mV in a culture environment, denitrification is expected to occur. However, a higher redox potential in the macro-environment does not necessarily reflect the redox potential within either the micro-environment of a biofilm or within an individual cell. Thus, denitrification may occur under apparently adverse or hostile conditions as indicated by the measurements performed on the macro-environment.

4.4.2.4 Growth Characteristics

Technical aspects of the different types of denitrification systems will be reviewed in later sections. However, as noted in the preceding section the cultural form does influence the requirements for denitrification and the efficiency of the process. One may be dealing with either freely suspended cultures or alternatively attached cultures in which the organisms adhere to solid support systems. Generally, the efficiencies of denitrification reactions using the different types of culture methods are very similar, i.e., approximately 90%. However, the rates of reaction can vary to a significant degree.

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Attached growth systems appear to offer the promising advantages of higher denitrification rates (as much as 10 times higher than suspended systems) coupled with low retention times - particularly in the case of fluidized-bed process (Jeris and Owens, 1975; Jeris et al., 1977; Stephenson and Murphy, 1980). This improved performance is attributed in part to higher cell masses in the system (compared to suspension systems), high mass transfer rates, large liquid/biofilm areas of contact, and uniform solids distributions.

The use of attached organisms does introduce some additional factors for consideration. In suspended systems, there is easy access of substrates to cells and thus it has been shown that the denitrification rate in such cultures will be independent of nitrate and carbon concentrations above a fixed saturation level, i.e., zero order (Christensen and Harremoes, 1977). In the case of biofilms, diffusion limitations are noted and it is proposed that the rate of denitrification is not zero order but rather half - order relative to substrate concentration (Stephenson and Murphy, 1980). However, the denitrification rate in biofilms is less sensitive to temperature changes than in the case of suspended growth systems.

The other important variable in denitrification i.e., pH, is also influenced by the use of attached growth systems. It has been demonstrated that in denitrifying biofilms, the pH in inner zones of the biofilm may be higher than the value in the macro-environment (Riemer and Harremoes, 1978; Arvin and Kristensen, 1982). This is attributed to diffusional restrictions on the release of carbonate and bicarbonates originating from the metabolism of carbon. It may also be influenced by the slow neutralization of hydroxyl ions produced as a by-product of the denitrification reactions. In suspended growth systems this alkalinity is neutralized by acidic constituents introduced in the feedstocks or present as by-products of other metabolic reactions, e.g., nitrification. However, as noted previously, the pH values of the macro-environment of a suspended growth culture may not reflect accurately the values in the micro-environment of the individual cell. Thus, the higher pH noted with the biofilms may also occur in certain cultural

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conditions at the cellular level within a suspended growth culture. The effects of these pH variations on denitrification rates are not well documented but operating problems have been reported such as the precipitation of calcium phosphate in the biofilms because of the higher pH values (Arvin and Kristensen, 1982).

The use of attached organisms will render a system less sensitive to inhibition by trace amounts of dissolved oxygen provided that the metabolic rate in the outer zones of the biofilm is sufficient to maintain anoxic conditions in the inner zones of the film. This will be assisted by nitrogen gases produced as by-products of the denitrification reaction. At the same time, the effect of diffusional restrictions on nitrogen removal from the inner denitrifying zones of the biofilms and hence on the rate and efficiency of denitrification requires further study (Harremoes et.al., 1980). This requirement also applies to the effect of these factors on the microbiological compositions and synergisms taking place within the biofilms.

4.4.3 Toxicants

Factors which will inhibit or be detrimental to the performance of a denitrification system are poorly documented. This is particularly true of mineral process streams. For this study, inhibition is regarded only from the standpoint of the overall conversion of nitrate to dimolecular nitrogen. Some inhibitors may inhibit specific metabolic reactions so that although nitrate reduction will occur, the end product will be an intermediate e.g. nitrite rather than gaseous nitrogen.

Known inhibitors of denitrification processes include sulphate (100-500 g/g), sulphide (1.28 mg S/g)(Tam and Knowles, 1979), acetylene (Balderston et al. 1976; Sorensen, 1978; Tam and Knowles, 1979), cyanide (Knowles, 1982; Knoetze et al., 1980), various pesticides (Knowles, 1982; Knoetze et al., 1980; Payne, 1983) and heavy metals (Holland, 1980; Bollag and Barabasz, 1979). The cyanide factor is of interest. It was found that the

toxic effect of cyanide is dependent on the carbon source used. Carbonyl compounds such as sugar or acetone appeared to convert cyanide ions to less harmful intermediate compounds which could be metabolized by the cells (Lewandowski, 1984). Thus denitrification still took place. With carbon sources such as methanol, denitrification was inhibited by cyanide ions.

Heavy metals are likely to be present in most mineral processing effluents. Since most processes tend to use mixed microbial cultures, it is difficult to relate the data from specific organisms to field situations. Francis and Hancher (1981) noted that nickel at a concentration of 0.5 mg/L, reduced a denitrification reaction by approximately 50%. This inhibition persisted for 8 days after which period the denitrification rate was restored. More significantly, the culture could then withstand nickel concentrations of approximately 4 mg/L without loss of denitrification. It would appear that a nickel-resistant culture had been enriched in the reactor.

When the denitrification process using this nickel-resistant culture was subsequently introduced at the Oak Ridge National Laboratory Y-12 Plant to treat effluents containing high nitrate concentrations as well as heavy metals (from the nuclear processing industry), denitrification was demonstrated. The conversion efficiency was high (more than 99%) but the rate was poorer than in pilot studies with synthetic feedstock. The process was inhibited by the accummulation of heavy metals in the sludge and a continuous process was therefore not feasible (Whinnery, 1981).

In the mining environment many organic and inorganic compounds and elements may be present. The sensitivity of denitrification processes to constituents such as heavy metals, lixiviants, anions etc., is still poorly documented and will require investigation at the laboratory level.

5. THE TECHNOLOGY OF BIOLOGICAL DENITRIFICATION

In this section, the operating characteristics of the different types of denitrification processes will be reviewed. Both attached growth and suspended systems have been used for denitrification. In either type of system, the gases generated as the products of the nitrate reduction can create operating difficulties and interfere with the rate of denitrification. Gases entrapped in the biomass can also result in problems at the clarification stage. Four types of system design or reactor will be reviewed namely suspended growth reactors, packed bed reactors, rotating biological contactors and fluidized bed reactors. The suspended growth reactor employs a subsequent clarification stage to separate and return biological solids to the process. The latter three reactor types involve attached growth of the organisms and may not require solids separation from the effluent.

The kinetics of biological reactions are usually expressed in terms of the Michaelis-Menten equations as shown below:

$$U_{dn} = U_{dn\ (max)} \frac{Cn}{K_{sn} + C_n} \cdot \frac{Cc}{K_{sc} + C_c}$$

Where U_{dn} = Actual denitrifier growth rate
$U_{dn\ (max)}$ = Maximum denitrifier growth rate
C_n = Concentration of nitrate
C_c = Concentration of carbon substrate
K_{sn} = Half saturation constant for nitrate
K_{sc} = Half saturation constant for carbon substrate

The half saturation constant is the concentration of a particular substrate at which microbial growth is one half of its maximum value for otherwise constant conditions (temperature, pH and other substrate concentrations). The half saturation constant for methanol as the carbon

sources (K_{sc}) has been determined to be 0.1 mg/L (U.S. EPA, 1975). This means that as little as 1 mg/L of methanol would result in a reaction rate of 90% of the maximum. Similarly, the half saturation constant for nitrate (K_{sn}) has been determined to be 0.16 mg NO_3-N/L. Denitrification is thus essentially independent of both the carbon substrate and nitrate concentration provided that there are no mass transfer limitations.

5.1 Suspended Growth Reactors

Suspended growth reactors for denitrification are generally designed under the assumption that the reduction of nitrate is essentially zero order with repect to nitrate when there is an excess of available carbon substrate. The rate of nitrate removal per unit of denitrifying biomass (or the unit denitrification rate) is thus used as a basis for the sizing of reactor vessels. The unit denitrification rate has been determined for the removal of nitrate for several sources of carbon. In one study, the rates of denitrification for 27 of 30 waste materials employed as carbon substrates were greater than or equal to that determined for methanol (Monteith et al. 1980). The study also noted that most of the wastes had substrate consumption ratios lower than that of methanol (Monteith, et al., 1980).

The temperature dependence of denitrification has been noted earlier. That dependence has been expressed in terms of an Arrhenius relationship, viz;

$$r_{dn} = 5.42 \times 10^{10} \cdot e^{-15300/RT}$$

where R = Universal gas constant

r_{dn} = Denitrification rate (Kg NO_3 -N removed/Kg biological solids / day)

T = Temperature, ^{o}K

The unit denitrification rate at 15°C is approximately 0.13/day (according to the above equation). A typical sludge concentration in a suspended growth denitrification system would be 2500 mg volatile suspended solids (VSS)/L. Hence, a denitrification rate based on the volume of the reactor would be in the order of 0.3 Kg NO_3-N/day/m^3 reactor volume.

Denitrification is considered (as noted above) to be a zero-order reaction with respect to nitrate concentration. The half-saturation constant, K_{sn}, used in the Michaelis-Menten equation is on the order of 0.1 mg/L, so the change to a first order reaction at low concentrations can be safely ignored. In a suspended growth system, no mass transfer limitations are expected and hence, the size of reactor is dependent upon the mass loading of nitrate to be reduced and is not influenced by its concentration. Subsequent clarification facilities are, however, very dependent upon the hydraulic loading.

5.2 Rotating Biological Contactors

Rotating biological contactors (RBC's) employ rotating media, generally disks, to support biological growth. RBC's were originally developed for the aerobic biological treatment of sewage. In that application, the multiple disks are approximately 40% submerged in the wastewater. Rotation of the disk serves several functions, including mixing of the wastewater, contact of the attached microbes with the organic substrate (i.e., the sewage) and oxygen, and removal from the disks and hence suspension of excess biological growth. Application of RBC's for the denitrification of wastewater has entailed the complete submergence of the disks in the wastewater.

RBC's have been widely employed for the aerobic treatment of wastewater to remove carbonaceous BOD and for nitrification. Commercially manufactured RBC's are typically up to about 4 metres in diameter and 10

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metres in length. For small installations, single RBC units may contain internal baffles to provide for a sequence of stages within the reactor. In larger installations, individual RBC units are used to provide the sequential stages.

If the biofilm of an RBC is not adequately penetrated by the organic substrate and nitrate, then the observed reaction will not be independent of either carbon or nitrate concentrations. Reports on denitrification in RBC's have not been consistent with respect to the observed relationships between denitrification rates and nitrate concentration. Murphy et al. (1977) and Autotrol Corporation (1978) claimed that on the bases of pilot-scale studies, the biological reduction of nitrate is independent of nitrate concentration down to 1 mg NO_3-N/L.

In contrast, Blanc et al.(1980), Cheung (1979), and Odegaard and Rusten (1980) have each reported that a first-order relationship exists between denitrification rate and the bulk liquid nitrate concentration. One recent review of the use of this promising technology for denitrification noted that further studies are required to "define design loading parameters that can be used with confidence" (Brenner et al., 1984).

Performances of RBC's used for denitrification have varied. Autotrol Corporation (1978) recomend a mass removal rate of 4.5g NO_3-N/m^2/day at 13°C. A full scale Autotrol unit was installed in 1976 for the treatment of leachate from a municipal solid waste landfill. This was a single, 3.6 m diameter, four stage RBC with a total of 8490 m^2 surface area. The design loading in this case was 7.4 g/m^2.day for wastewater at 15°C and the use of methanol as the carbon source. On a volumetric basis, the design loading was equivalent to 436g NO_3-N/m^3/day. When treating relatively low strength nitrate wastewaters (17-25 mg NO_3-N/L) no subsequent clarification may be necessary (Murphy et al., 1977). The RBC by its nature requires no backwashing or recycle.

5.3 Packed Bed Reactors

Packed bed reactors of several configurations and a variety of biomass support media have been used for denitrification. The fixed support generally is either a highly porous, synthetic media or a low porosity, granular media. Both upflow and downflow systems have been demonstrated. Provision must be made for backwashing and treatment of backwash water for both types of packed bed reactor.

5.3.1. High Porosity/Large Media

Systems using high porosity media have generally used either bundled, corrugated sheet or randomly packed plastic material similar to that which is employed with conventional trickling filters in sewage treatment processes. High void ratios (90-95%) in the filters reduce the potential for fouling due to biomass growth. Excess biomass is either allowed to slough off (as in a trickling filter) or is removed by infrequent backwashing. In one full-scale operation, where plastic ring shaped media were used, backwashing once every four weeks was found to be sufficient (WPCF, 1983). Both air and water were used at rates of 6.8 $L/m^2/s$ and 600 $L/m^2/s$, respectively. Without periodic backwashing, high levels of effluent suspended solids may be experienced (WPCF,1983).

Kinetic studies have been conducted for a packed bed using 1.9 - 3.8 cm diameter plastic spheres (Jewell and Cummings, 1975). This work noted a dependence upon effluent nitrate concentration for the denitrification rate. For operation at 20°C, a maximum reaction rate of 0.45 Kg NO_3-N/Kg VSS/day and a half saturation constant (K_s in the Michaelis-Menten equation) of approximately 20 mg NO_3-N/L were determined.

The temperature dependency of the surface removal rate for denitrification in a high porosity packed bed reactor has been determined and is noted in the following Arrhenius equation (WPCF, 1983):

$$R_{dn} = 441\ e^{-11.09/RT}$$

where R_{dn} = Surface denitrification rate, $Kg/m^2/day$
R = Universal gas constant
T = Temperature, ^oK

5.3.2. Low Porosity/Fine Media

Most work with low porosity/fine media systems have employed granular material in the range of 2-5 mm in diameter (WPCF, 1983). Use of media in this size range will affect filtration as well as nitrogen removal. This feature does, however, necessitate frequent backwashing to reduce head loss associated with the build-up of suspended material.

Nitrogen gas build-up has also been noted with this type of reactor. Backwash rates of 5.4 to 10.8 $L/m^2/s$ for one to two minutes, every 4 to 12 hours have been found to be effective in controlling gas and solids accumulation (U.S. EPA, 1975). With effective backwashing, effluent solids normally can be maintained at less than 10 mg/L (U.S. EPA, 1975).

One report of downflow packed beds (of 2-5 mm rounded sand) has studied the kinetics of denitrification (Harremoes and Reimer, 1977). They noted that at nitrate concentrations below 30 mg NO_3-N/L the effects of pore diffusion result in an overall reaction rate proportional to the square root of the nitrate concentration, as noted in the following expression;

$$r_a = k_{1/2}\ C^{1/2}$$

where r_a = Surface denitrification rate, Kg NO_3 $-N/m^2.d$
$k_{1/2}$ = Half order reaction rate constant

(0.48 $mg^{1/2}/d.m^2.min$ @ 10^oC)

C = Nitrate concentration, mg NO_3/L

The temperature dependence of the half rate constant is determined as noted below;

$$k_{1/2}.t = k_{1/2}.10 \times 10^{0.022(t-10)}$$

where t = Temperature, ^{o}C.

5.4 Fluidized Bed Reactors

Fluidized bed denitrification systems employ a fine support medium (often sand, coal or activated carbon) which is suspended of fluidized by the upward flow of liquid. The total surface area of the fluidized particles is available for biological growth and, since the system is inherently resistant to fouling, fine particles with a very high specific area may be used to support biological growth. This can result in biomass concentrations in the order of 18,000 to 40,000 mg volatile solids/L (WPCF, 1983).

Fluidization and hence bed expansion, is accomplished by superficial velocities in the order of 1 to 2 cm/s (Stephenson and Murphy, 1980; Pitt et al. 1979). In this type of system the support media are effectively being continuously backwashed. When high strength nitrate wastes are being denitrified, treated effluent must be recycled to provide the hydraulic throughput required to suspend the media.

One full scale fluidized bed for denitrification of municipal wastewater has been developed for a municipality in Florida (WPCF, 1983). The design incorporates four 5.8 by 5.8 metre reactors to treat 91,000 m^3/day of wastewater.

Particles laden with biological solids must be removed periodically from the denitrification fluidized bed. The biological solids are then removed from the media particles by hydraulic or mechanical agitation.

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A portion of the support particles will be lost from the fluidized bed continuously as a result of abrasion. In one pilot-scale system using coal particles, 5% of the support media inventory was lost per week (Pitt et al., 1979).

Some operational difficulties may be experienced in the treatment of calcium-bearing wastewaters through the formation and accumulation of $CaCO_3$ and hence changes to the fluidization properties of the media (Francis and Hancher, 1981).

The kinetics of fluidized bed denitrification have been studied by several investigators who employed different techniques and reached, to some extent, similar conclusions. Pitt et al. (1979) examined the influence of nitrate concentration upon the removal rate of nitrate in a fluidized bed (coal particles) reaction. They found that the reaction rates could be expressed by the Michaelis-Menten equation. Values for maximum rate (R_{max}) and half saturation constant (K_{sn}) were 146 g NO_3-N/l · d and 680 mg NO_3-N/L respectively. They also noted that the reaction rate data could be empirically expressed in terms of half-order kinetics, with a rate constant (K 1/2) of 43 $(g/L)^{1/2}$. The relevent equations are shown below:

Michaelis-Menten Equation:

$$R = R_{max} \cdot \frac{Cn}{K_{sn} + C_n}$$

where R = Reaction rate
R_{max} = Maximum reaction rate
Cn = Nitrate concentration
Ksn = Half-saturation constant

Half Order Kinetics Equation:

$$R = R_{1/2} C_n^{1/2}$$

Where $R_{1/2}$ = half-order reaction constant

Stephenson and Murphy (1980) compared alternate kinetic models for fluidized beds using sand as the support media. They found that a half order kinetic model best fit their data. Mulcahy et al. (1980) have presented a detailed model of the mass transfer and intrinsic kinetics of denitrification in fluidized beds and concluded that a nitrate concentration below 36.6 mg NO_3-N/L the reaction will be 0.45 order with respect to nitrate.

Stephenson and Murphy determined the effect of temperature on the half order rate constant as shown in the following equation:

$$R_{1/2} = 0.78 \ e^{-8763(1/T - 1/288)/R}$$

where $R_{1/2}$ = Half order rate constant, $(mg/L)^{1/2}/min)$
R = Universal gas constant
T = Temperature, ^oK

The thickness of the biomass film on the support media particles can have a strong influence on the performance of fluidized bed denitrification reactors. Excessive biomass accumulation can cause difficulties with particle adhesion and channeling if the biomass reaches 20% of the support particle mass (Pitt et al., 1979). Mulcahy et al. (1980) have predicted that if the biomass film thickness exceeds the particle radius by 15% the bulk liquid nitrate will fail to fully penetrate the biofilm and reduce the effectiveness of the process.

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Williamson and McCarty (1976) have suggested that the carbon substrate (methanol) may be the limiting factor in biofilm processes. The diffusion coefficient for methanol is twice that of nitrate which, together with other factors, means that methanol concentration must be five times that of nitrate if it is not to be flux limiting. Gauntlett (1981) came to essentially the same conclusion, noting that fluidized bed denitrification rate was independent of nitrate down to 1 mg NO_3-N/L and methanol to 5 mg/L.

6. REVIEW OF SELECTED APPLICATIONS OF BIOLOGICAL DENITRIFICATION OF POSSIBLE SIGNIFICNCE TO THE MINING INDUSTRY

In this section, we will assess the technical feasibility of applying biological denitrification in the mining environment. There are, however, no known cases where denitrification has been used for nitrate removal at a mine site. There are also only few documented applications of this type of process to industrial effluents. Three industrial installations will be reviewed. This will be followed by an assessment of a selected group of processes for treating high nitrate containing wastes. The wastes in question resembled mining effluents or process solutions in their composition i.e., metals etc., or alternatively, the process in question is considered to offer some potential advantages for a mining application.

6.1 The Treatment Plant at the Maitland Works of Dupont of Canada

A biological treatment plant for nitrification/denitrification and BOD reduction of wastewater from Dupont's Maitland Works has been in operation since 1973. The wastewater, which derives principally from the manufacture of nylon intermediates, contains nitric and nitrous acids, organic carbon, ammonia and organic nitrogen compounds. The composition is as follows:

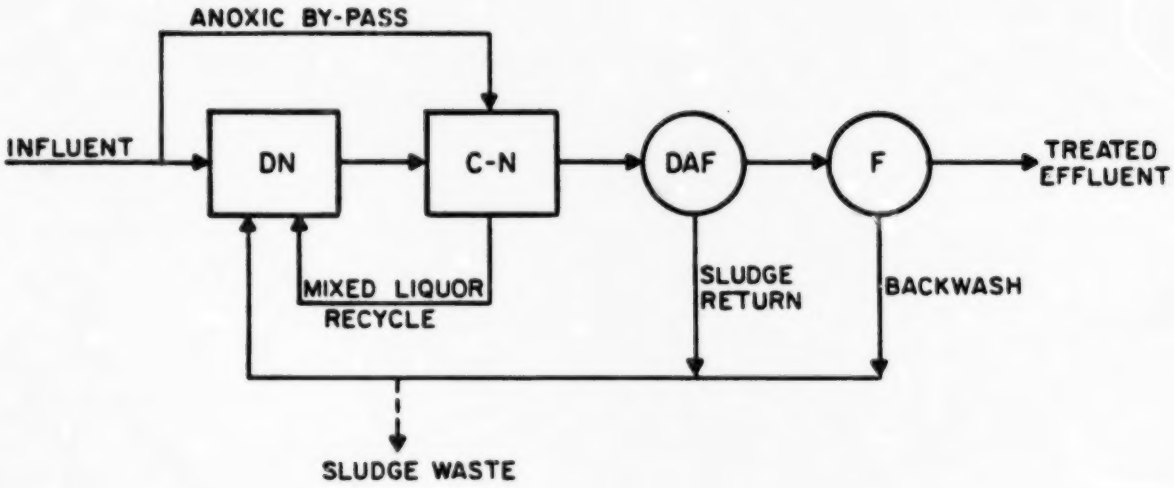
	Mean (mg/L)	Variability (from 24h composites)
TOC	750	x1.38
BOD	1500	x1.38
NO_T	160	x2.49
TKN	150	x2.04
NH_4	75	x2.04
pH	3 - 5	

TOC = Total organic carbon
BOD = Biological oxygen demand
TKN = Total Kjeldhal nitrogen

The plant was installed following three years of laboratory and pilot plant studies and is a single-sludge suspended growth system consisting of an anoxic pre-denitrification basin followed by an extended aeration basin. Sludge separation is accomplished in a dissolved air flotation unit and a dual-media filter (Figure 1) (Climenhage, 1972, 1973; Cooper et al., 1977a, 1977b; Bridle et al., 1979).

Solids retention time in the system is varied from 45 to 80 days resulting in equilibrium volatile suspended solids (VSS) levels of 2000 - 3500 mg/L in the basins.

In this system, the denitrification reactor handles both the incoming waste and the nitrified wastewater returned from the aerobic stage. Influent flow to the denitrification reactor is 11,000 m^3/day. Hydraulic retention time (HRT) in the anoxic basin, based on feedrate, is 36 hours. Denitrification is reported to be almost complete, with better than 98% removal of nitrate + nitrite being achieved. Twenty to thirty per cent of the organic carbon is removed in the denitrification stage. Contained organic carbon is adequate for the denitrification and there is no external carbon addition.



LEGEND : C-N - CARBON REMOVAL AND NITRIFICATION
DN - DENITRIFICATION
DAF - DISSOLVED AIR FLOTATION
F - FILTER

Fig. 1 FLOW DIAGRAM FOR THE DuPONT-MAITLAND TREATMENT PLANT. (Bridle, 1979)

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The pH in the anoxic basin remains consistent between 6.5 and 7.5, in spite of the low pH of the feed solution. This consistency is attributed to the neutralizing effect of either the alkalinity generated in the course of nitrate reduction or to the removal of acidic carbon compounds. Similarly, a buffering effect is attributed to the mixed liquor being recycled from the aerobic basin. The addition of neutralizing chemicals is rare.

Upsets occur only infrequently and generally result from plant spills. This "shock" to the system moves the anoxic pH out of its specified range i.e., to either less than 6.5 or greater than 9.0. Recovery is achieved by pH adjustment or by diversion of feed solution.

The aerobic basin HRT, based on feed rate, is 120 hours. Denitrification waste overflows the anoxic basin to the aerobic basin, where the remaining carbon is oxidized and ammonia and organic nitrogen are converted to nitrate. Mixed liquor recycle to the anoxic basin, at rates of up to twice the feed rate, allows for denitrification of the waste to proceed and hence ensures a low nitrate concentration in the final effluent.

Some denitrification has been observed to occur in the aerated basin. Dissolved oxygen (DO) profiles in this stage indicate levels of 3.5 mg/L of O_2 at the aerobic outlet with as low as 0.5 mg/L O_2 in the zone around the anoxic basin outlet. The denitrification probably occurs in the low DO zone and results from either there existing a zone of poor mixing or alternatively the oxidation rate is so rapid as to exceed the dissolution rate of oxygen into the liquid.

Considerable difficulties were experienced with the nitrification stage. Factors such as shock loadings, inadequate aeration and low temperatures during the winter months were all encountered. However, the denitrification unit appears to have operated efficiently at all times.

6.2 National Lead Company

The National Lead company is in the proceess of constructing a fluidized-bed biological denitrification system to treat a high nitrate effluent at the rate of 100,000 gallons per day. This follows development and testing of the system at the Oak Ridge National Laboratory (ORNL) (Patton, 1984).

The system will comprise four columns, to be operated in series. each column is 4 ft. in diameter with a fluidized bed height of 38 ft. The top of the column is flared and contains a 7 ft. diameter section to disengage solids. The total column height is 52 ft.

Fine coal (nominally 60 mesh) will be used as a medium for bacterial growth. Each column is to be operated with a vibrating screen fitted with a 60 mesh deck, which will be used to remove excess sludge and return coal to the column. The entire effluent stream from each column will pass over the screen. About 5% of the coal inventory is expected to be recovered on the screens each week.

Treated effluent is to be recycled for feed dilution so as to maintain feed nitrogen concentration between 2000-3000 mg/L. The undiluted wastewater contains up to 5000 mg/L of NO_3-N. Total system water retention is about 2 hours based on new feed plus recycle. Methanol will be addded as a nutrient for bacterial growth. The C:N ratio of the feed will be maintained at 1.2 - 1.4 : 1.0.

The wastewater will be pretreated with lime at pH 10 - 11 to precipitate and remove toxic heavy metals. It is anticipated that the biomass will adsorb residual heavy metals, particularly copper and chromium, without any deleterious effect. System pH will be controlled at about 7.0 by the addition on nitric acid. The system is expected to reduce the nitrate nitrogen concentration in the treated effluent to approximately 100 mg/L, which is equivalent to a removal efficiency of approximately 98%.

6.3 The Chemical Works of DSM (Netherlands)

A new wastewater treatment system was designed for the above plants (Baenens, 1980). The plant complex at Geleen in the south of the Netherlands generates a wastewater containing organic nitrogen, ammonium ions, urea, nitrite and nitrate ions. A representative sample of the wastewater being treated at the plant will contain:

	Concentration (mg/L)
COD	55
TKN	20
NH_4 -N	5-10
NO_3 -N	100-125

COD = Chemical oxygen demand

The process schematic for this three-stage combined nitrification/denitrification system includes a pre-denitrification stage, a nitrification-biological oxidation reactor, and a final denitrification step. The plant consists of three parallel process streams. The scale of operation in each stream is:

Pre-denitrification - 13000 m^3
Nitrification - 37000 m^3
Final denitrification - 4500 m^3

The maximum loading of wastewater on the total system (three streams) is 4500 m^3/h.

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As an integrated system, it is difficult to extract data from the published information to evaluate individually either the nitrification or denitrification process. A few important aspects can be identified. The use of the pre-denitrification stage ensures that maximum nutrient value is gained from the carbon content of the feed solution. The recycling of the water from the nitrification reactor is intended to make sure that an excess of nitrate is maintained relative to the carbon content in the pre-denitrificaaion stage. An external source of BOD is required for the final denitrification process. Methanol was found to be too expensive for practical application. A wastewater stream (from a phenol plant) containing predominantly acetic and formic acids proved to be an effective substitute carbon source.

Most of the start-up problems appear to have originated in the nitrification reactor. The only concern with the denitrification reaction was the possibility of its inhibition by cyanide ion. This proved to be unfounded due, possibly, to better than expected control measures at the plant process level.

6.4 Studies/Systems Related to Possible Mining Problems

As noted above, the specific application of denitrification to the treatment of industrial wastewaters has been a relatively rare occurrence. The majority of the process development in this field has been directed at municipal wastewater treatment systems where large daily volumes of wastewater and high carbon loadings are the norm. Nitrate concentrations, relative to the concentrations of other nitrogen compounds (e.g., ammonium) are usually low and the ratio of total carbon/total nitrogen tends to be high. Although the low nitrate concentration may match the situation in many mining effluents, the latter group are characterized by usually a low carbon content as well as higher concentrations of toxic metals relative to biomass. Similarly, large scale treatment facilities would not be an economic proposition for nitrate removal in the mining industry. Systems based on energy substrates other than carbon (e.g., autotrophic denitrifying organisms using reduced sulphur compounds) or systems designed to handle high nitrate

concentrations (e.g., possibly greater than 50 Kg NO_3-N/m^3 in isolated process effluents from the nuclear industry) may match better the perceived needs of the mining industry. Such needs might include the treatment of isolated process effluents of smaller volumes but high nitrate concentrations. We will review briefly some examples of such systems.

6.4.1. Systems Handling High Nitrate Concentrations

In this section we will consider systems handling high nitrate concentrations other than those based on autotrophic organisms. The source of most of these high nitrate effluents will be either the nuclear industry or the fertilizer industry. Both types of effluent will commonly contain concentrations of heavy metals which will potentially inhibit the denitrification process. Compared with nitrifying organisms, the denitrifiers are less sensitive to toxic metals. However, this tolerance is limited and at the concentrations which may be present in industrial effluents, caution must always be used that a limit of tolerance is not exceeded. In the development of a system to treat nuclear industry effluents containing nitrate concentrations as high as 50kg NO_3-N/m^3, Francis and Hancher (1981) noted that very low concentrations of nickel (e.g., less than 0.5mg/L) were strongly inhibitory on the denitrification reaction of a microbial culture isolated from an estuarine environment. However, acclimation of the culture did develop presumably due to the enrichment of nickel-tolerant species in the culture. Thus nickel concentrations of approximately 4 mg/L could be tolerated without detrimental effects on denitrification.

Francis and Hancher (1981) selected a fluidized bed reactor (FBR) using coal particles as support media for the treatment of these high nitrate wastes. Factors such as the salts used to neutralize the effluents (originally nitric acid) and the nature of the carbon source were all found to be critical. Thus, the use of caustic soda to neutralize the feed solution resulted in significant reduction of denitrification rates particularly at higher nitrate concentrations. Nitrite tended to accumulate in the cultures which suggested that the presence of sodium inhibited the nitrite reduction reaction. When calcium was used to neutralize the effluents, the

denitrification rate was also reduced as the nitrate concentration was increased. This inhibition was believed to be due to phosphate starvation of the organisms arising from the precipitation of calcium phosphate. An additional problem with the use of lime to neutralize the incoming effluent was the accumulation of calcium carbonate and the resultant detrimental effect on the reactor performance.

Carbon substrates must be added at high concentrations to maintain the ratio for C:N of 1.3 - 1.5. In bench tests (tapered FBR, 2.54cm at base to 5.08cm diameter at top)and using a fixed nitrate concentration, methanol did not inhibit the denitrification in an FBR up to concentrations of 23Kg methanol-C/m^3. Calcium acetate on the other hand was inhibitory at concentrations of 6Kg C/m^3. This may again indicate a phosphate limitation effect. Methanol or ethanol were subsequently used preferred carbon sources by the authors. No data were provided on the tolerance to methanol at higher nitrate concentrations.

This process design was subsequently tested at pilot scale to treat nitrate wastes from solvent extraction raffinates at the U.S. Department of Energy's Portsmouth, Ohio gaseous diffusion plant (Francis and Hancher, 1981). Two FBR's were used in series (each 20 cm ID x 7.5 m length) to treat influent solutions containing up to 2.5Kg NO_3-N/m^3. Using sodium hydroxide to neutralize the acidic influent, an incoming nitrate concentration of 0.25Kg/m^3 was reduced to less than 10g (NO_3 + NO_2)-N/m^3 in the final effluent. At the higher NO_3-N concentration of 2.5Kg/m^3, the final effluent still contained 1.6Kg NO_3-N + 0.12Kg NO_2-N/m^3.

This system was also introduced at full scale at the Oak Ridge National Laboratory Y-12 plant. However, problems were encountered when the system was operated on a continuous basis. Heavy metals in the system apparently concentrated by bioadsorption on the microbial surfaces and led to inhibition of the denitrification reaction (Whinnery, 1981).

6.4.2 Autrophic Denitrifying Systems

As noted above, in situations where there is an inadequate source of carbon in an effluent, an external carbon source must be added to maintain the efficiency of the denitrification. However, the supplying of an energy source such as methanol is an expensive proposition. Various studies have indicated that other carbon sources such as industrial wastes can be substituted for the methanol provided that they are readily degradable. In the case of a mine site there is still the cost of transporting the carbon source to an isolated area. One solution to this economic problem is the use of autotrophic denitrifying bacteria.

Thiobacillus denitrificans will reduce nitrate to nitrogen while at the same time using reduced sulphur compounds as energy substrates. The organism gains its energy from the oxidation of compounds such as elemental sulphur, sulphides, sulphite and thiosulphate etc. The organism uses inorganic carbon compounds as its source of cellular carbon. It has been claimed that autotrophic denitrification with T. denitrificans in either suspended or attached growth is as effective as denitrification by heterotrophic organisms (Steinmuller, 1981; Steinmuller and Kutzner, 1980). In a packed bed reactor consisting of a 1:1 mixture of sulphur and dolomite, an effluent containing 40 mg NO_3-N/L was almost completely denitrified. The reaction kinetics were stated to be close to first-order at the nitrate concentrations investigated (Sikora and Keeney, 1976). In a similar experiment using a packed bed and marble chips media, effluents were denitrified using thiosulphate or sulphur as nutrients sources (Yamauchi et al., 1981)

The major product of the cells' metabolism, other than gaseous nitrogen, is sulphate. The production of sulphate ion in a packed bed reactor was approximately inversely proportional to the decrease of the nitrate plus nitrite nitrogen concentration (Sikora and Keeney, 1976). Another byproduct

of the sulphur reduction will be acid. However, a significant portion of this acid will be neutralized by the alkali generated during the nitrate reduction. The use of dolomite as a support material by Sikora and Keeney (1976) may offer the dual benefit of neutralization of the acid by reaction with the carbonate while at the same time the carbon dioxide produced will act as a carbon source for the organism.

This approach does not appear to have been evaluated at pilot scale level. Sources of reduced inorganic sulphur compounds will be plentiful at most mine sites and could represent a very inexpensive energy source. However, the sulphate generated in the reaction may have to be recovered by precipitation. While still not a well developed technology, the use of autotrophic organisms deserves further investigation. Temperature will be a critical variable and currently we are not aware of how significant will be the effect of low temperatures on the rate of denitrification.

7. ALTERNATIVE PROCESSES FOR NITRATE REMOVAL

A number of other authors have recently investigated alternative denitrification procedures and their relative costs. Gregory and Sheiham (1981) compared the economics of biological fluidized-bed denitrification for nitrate removal from drinking water with ion exchange, diluting with water from another source and the provision of storage facilities to retain water from the same source. They concluded that the fluidized-bed system was the most cost effective over a wide range of scale of application. Ion exchange and substantial replacement by water from another source within a distance of 10 Km compared favourably when the supply was 4500-5000m^3/day but economy of scale was more significant in the case of fluidized-bed treatment applied to larger systems. This did not apply to operating costs because of the complexity of the external carbon dosing system and the cost of the chemical.

Guter (1982) evaluated physical/chemical methods including reverse osmosis, ion exchange and a combination of the two procedures for the removal of nitrate and nitrite from well water. He found that ion exchange

was the preferred physical technique. In another study with potable water, physical/chemical procedures to remove nitrate were compared with both autotrophic and heterotrophic biological denitrification. It was concluded that autotrophic denitrification was the most suitable process.

These tests with drinking water permitted some comparative evaluations of different techniques using a standardized substrate. In the case of mining and mineral processing effluents, one might expect additional problems with techniques such as reverse osmosis and ion exchange. The presence of competing anions will be more significant in the case of minewaters. This will result in greatly increased costs for chemicals to elute and regenerate the resin. One solution to this problem may be the development of semi-liquid ion exchange materials with a preference for nitrate over ions such as chloride or sulphate. It was stated that alkylated amines will preferentially adsorb nitrate from a mixed nitrate/chloride solution by a factor of twenty. Its preference for sulphate and bicarbonate was even greater (Grinstead, 1971). Nevertheless, an operating problem likely to be present in the mine environment for ion exchange processes as well as reverse osmosis processes will arise from the presence of microbial slimes or iron precipitates. Such problems are expected to be significant. The use of biological denitrification with potable water purification may cause some misgivings to the water treatment engineers who may be loath to add carbon to an already relatively pure solution. For the mine engineer, such reservations need not be so profound and biological denitrification processes look promising for situations requiring nitrate removal.

Pitt et al. (1979) compared nitrate removal by fluidized-bed biological denitrification, ion exchange and a chemical treatment using sugar. The estimates were based on treating 1×10^5 gal/day containing 1 g NO_3/L. The fluidized-bed denitrification was the more efficient treatment method and the by-product, i.e., biomass, was less of a disposal problem. The economics for ion exchange were similar to the biological treatment but the

former method leaves a concentrated nitrate solution to be disposed of when the resin is regenerated. The chemical procedure not only appeared uneconomical but also generated a waste stream which was still high in nitrate and carbon.

Numerous studies have been conducted to compare the alternative biological denitrification processes and several of these have used uranium processing effluents for evaluation (Francis and Hancher, 1981). A wastewater containing approximately 1g NO_3-N/L was denitrified in a fluidized-bed reactor, a stirred bed reactor and a continuous-flow stirred reactor. The fluidized-bed reactor was reported to be 10-15 times more efficient than the stirred-bed reactor and about 50 times more efficient for nitrate removal than the continuous-flow stirred system.

In a similar study, a uranium processing raffinate was denitrified with an efficiency of 99.6%. The NO_3-N concentration in the final effluent was 100 mg/L. However, the rate of denitrification achieved in this system was only about one third that of a fluidized-bed reactor used for treating a similar effluent at the Oak Ridge National Laboratory Y-12 Plant.

An alternative biological denitrification approach uses algal species. These intensive algal harvesting techniques with organisms such as Chlorella vulgaris (Przytocka-Jusiak et al., 1984) or undefined algal species (Hendricks and Bosman, 1980) use the sun as their energy source and the nitrate and other nitrogen compounds are assimilated and converted to cellular biomass. In addition to requiring a source of nutrients such as phosphorus, such processes will require large culture surfaces or basins, and harvesting techniques. The production of a solid product rather than a gaseous product, as is the case with the microbial denitrification systems, will be a considerable nuisance. Finally, it is doubtful that the temperatures and light intensities at most mine sites in Canada would be adequate for intensive algal culture.

Based on the various evaluations which have been reviewed, it would generally appear that biological denitrification processes are the more practical and more economical alternatives. Similarly, it would appear that attached growth systems are more efficient than suspended growth processes. It is important to note that the majority of the studies have been conducted on domestic wastewaters rather than mine effluents. In the latter case the economics may not be so favourable. However, based on current knowledge, it is reasonable to believe that the findings would apply to mining effluents.

8. RELATIVE PROCESS ECONOMICS

To arrive at realistic cost data on the different types of biological denitrification processes is difficult. The majority of the information has been derived from combined nitrification/denitrification processes for the removal of nitrogen from domestic wastewaters. Estimations for the Maitland plant also involve a nitrification stage and hence it is not possible with the data available to isolate the economic data specific to the denitrification stages.

Other comparative cost estimates for different nitrate-removal techniques have been prepared (Pitt et al. (1979); Gauntlett, 1981; Bosman and Hendricks, 1981; Gregory and Sheiham, 1981). Data are presented based on 1976-1981 values in currencies including U.S. dollars, pounds sterling, and rands (South Africa). The data also poorly match with regard to the volume of effluent treated per day, the concentration of nitrate on which the calculations are based, and the concentrations of potential interfering anions. Calculations to convert the data to a standard unit for ease of comparison would require a large number of assumptions to be made.

Accordingly, it is considered more relevant to express the cost data from each source on a relative scale. Thus, the lowest cost procedure in each study has been given the rating of 1.0. The ratio of each of the other cost estimates in that study is then calculated (Table IV).

TABLE IV: RELATIVE ECONOMICS OF SOME NITRATE-REMOVAL TECHNIQUES

	BIOLOGICAL DENITRIFICATION				
Reference	SGR (1)	FBR (2)	Ion Exchange	Reverse Osmosis	Chemical
Bosman & Hendricks (1981)	1.0		1.4-1.6		
Gauntlett (1981)		1.0	5.7		
Pitt et.al. (1979)		1.0	1.1		3.4 (3)
Gregory and Sheiham (1981)		1.0	1.2-10.3(4)		4.5-5.7(4,5)
Jeris and Owens (1978)	3.6	1.0			
Sorg. (1980)			1.0	1.1-3.6	
O'Brien (1983)			1.0	6.9-9.2	

(1) Suspended Growth Reactor
(2) Fluidized Bed Reactor
(3) Sugar Reduction Method
(4) Dependent on Scale of Operation
(5) Coagulation/Sedimentation

Based on these studies, biological denitrification in a fluidized-bed reactor is the more economically attractive procedure. Cost data for RBC denitrification processes have not been obtained but it is anticipated that the economics will compare well with those of fluidized-bed treatments.

9. CONCLUSIONS AND RECOMMENDATIONS

Biological denitrification is a well established process. As a combined process with the nitrification reaction, it is widely used to remove total nitrogen contaminants from domestic wastewater. Removal efficiencies in excess of 95% are common. These combined processes are also being introduced in a variety of industrial applications and a process to specifically remove nitrate from a minerals plant effluent is being installed.

Biological denitrification for the removal of nitrate and nitrite from mining and mineral processing operations is technically feasible. Nitrification, on the other hand, is the more troublesome sector of the combined nitrification/denitrification processes. Nitrification is highly sensitive to both temperature fluctuations (particularly temperatures less than approximately 15 ^{o}C) and the presence of heavy metals. Its use in the mining or mineral processing environment is not recommended. Denitrification is a more robust process. The reaction will function at low temperatures and constant operation at low temperatures will probably result in the selection of a psychrophilic bacterial population. The nitrate-reducing organisms are also more tolerant than the nitrifiers to the presence of heavy metals and other toxic inorganic species such as cyanide. Chemical pretreatment might be required to remove higher concentrations of these toxicants which otherwise could lead to a loss of efficiency in the denitrification reaction.

The attached growth fluidized-bed process is now considered to be proven technology. This system offers the lowest operating costs of the alternative processes, higher reaction rates, improved protection against toxicants and is less sensitive to temperature variations.

A close competitor is the rotating biological contactor (RBC). It provides all of the safeguards and advantages of the fluidized-bed process and its covered mode of operation also assists in the maintenance of suitable operating temperatures. This may be a critical factor in applying denitrification processes in the colder mining regions of Canada. Although mechanical factors limit the size of individual RBC units, larger volumes of effluent can be accommodated by arranging units in series or parallel.

Physical/chemical denitrification processes for the removal of nitrite or nitrate are limited to ion exchange and advanced treatment methods such as electrodialysis and reverse osmosis. The two latter processes are not suited to the treatment of large volumes of effluent, and all three processes are expensive to operate. Biological denitrification may be a more economical route for nitrate removal. Although the requirement for nutrients such as carbon may create problems and potential economic limitations at isolated mine sites, the biological systems offer considerable flexibility in design. Thus, the sewage generated on-site may be an important nutrient source which can limit the amount of external carbon source which has to be transported to a site. The total carbon requirements will also depend on the volume of effluent which has to be treated. Minimizing the volume of effluent or process stream to be treated will reduce to some extent the total carbon requirement. However, since the total carbon requirement is a relatively fixed value relative to the nitrate concentration present, the major benefit from reducing the volume of effluent to be treated will be in the size of treatment system needed and hence the capital cost.

An alternative to carbon based processes will be the use of autotrophic organisms. Additional studies will be required to assess how sensitive is the denitrification rate on the temperature and nitrate concentration etc.

The lack of mining applications and the corresponding lack of extensive data on the nitrate and nitrite concentrations in Canadian mine and mill effluents both reflect the relatively limited concern at both the industry and regulatory levels as to the need to treat mining industry effluents to remove nitrate. The data on nitrate discharges from representative mines across the country are very limited. The general indication is that unless more stringent objectives are introduced, there does not appear at present to be a widespread need for nitrate removal technologies to meet health related standards. There may, however, be some local, specific situations which do not fall within this category.

Of greater concern may be the contribution of nitrate discharges to localized environmental problems such as eutrophication, algal growth etc. Such problems will be the result of several factors and the nitrate concentration in the effluent need not necessarily be in excess of a stated standard. The dilution factor at different times of the year in the watersheds into which an effluent is being discharged may be a cause of the problems. Similarly, the development of eutrophication will also depend on the presence of other nutrients such as phosphorus. Since no two effluents and locations are identical, the identification of problems cannot depend solely on the monitoring of the nitrate concentrations in effluents.

The course of action recommended is as follows:

- Update the information base on nitrate concentrations in effluents. The survey should include ammonia and nitrite concentrations, i.e., those species which can be converted to nitrate or which on their own can contribute to environmental problems such as eutrophication.

- Assess the level of occurrence of environmental problems such as eutrophication in receiving waters at mine sites and relate them to the nitrogen discharge. Particular attention should be paid to indirect sources of nitrogen such as explosives.

- In those cases where nitrate and possibly total nitrogen levels are unacceptable both in terms of discharge level and receiving water quality, investigate the on-site sources of the contaminants and hence the possible alternative treatments.

- Mount a test program to investigate the applicability and feasibility of using biological denitrification for the removal of nitrite and nitrate nitrogen from mine effluents. This would involve selecting the technology to be evaluated and then assessing its performance with effluents from mines with demonstrated problems due to nitrate discharge. Since no single mine effluent would be typical of the industry, the program should incorporate the testing of effluents from different trouble spots. This evaluation would include not only the denitrification efficiency but also the availability of nutrients and the cost factors.

- Denitrification should also be considered from the standpoint of generating on-site a biomass able to remove trace concentrations of heavy metals from effluents.
  The metal complexing ability is limited but the use of biological denitrification could provide two benefits to the mining industry.

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